THE JOURNAL OF EXPERIMENTAL MEDICINE

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME THIRTY-FIFTH

WITH SIXTY-SEVEN PLATES AND
ONE HUNDRED AND THIRTY FIGURES
IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1922

COPYRIGHT, 1922, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

JAN 3 1922 Medical Lib.

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME XXXV, No. 1 JANUARY 1, 1922

PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1922, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of between 500 and 600 pages. The subscription price is $3.00 per volume to subscribers in the United States and Canada, payable in advance; $3.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

Med.
Wehr

CONTENTS.

No. 1, January 1, 1922.

PAGE

Olitsky, Peter K., and Gates, Frederick L. Experimental studies of the nasopharyngeal secretions from influenza patients. VI. Immunity reactions..... 1

Stillman, Ernest G. The frequency of *Bacillus influenzæ* in the nose and throat in acute lobar pneumonia..... 7

Carrel, Alexis, and Ebeling, Albert H. Heterogenic serum, age, and multiplication of fibroblasts..... 17

Pearce, Louise, and Brown, Wade H. A study of the relation of *Treponema pallidum* to lymphoid tissues in experimental syphilis..... 39

Gates, Frederick L. Studies on agglutination with the aid of the centrifuge. The influence of temperature on absorption and flocculation..... 63

Drinker, Cecil K., Peabody, Francis W., and Blumgart, Herrmann L. The effect of pulmonary congestion on the ventilation of the lungs. Plates 1 to 3..... 77

No. 2, February 1, 1922.

Auer, John. Experimental generalized analgesia after exposure to some war gases. Plates 4 to 6..... 97

Olitsky, Peter K. Experimental studies on the etiology of typhus fever. II. Survival of the virus in aerobic and anaerobic culture media..... 115

Olitsky, Peter K. Experimental studies on the etiology of typhus fever. III. Filtration experiments..... 121

McMaster, Philip D. Do species lacking a gall bladder possess its functional equivalent?..... 127

Robertson, Oswald H., and Rous, Peyton. Sources of the antibodies developing after repeated transfusion..... 141

NYE, ROBERT N. Studies on the pneumonic exudate. V. The relation of pneumonic lung protease activity to hydrogen ion concentration, and a consideration of the origin of the enzyme..... 153

LITTLE, RALPH B., and ORCUTT, MARION L. The transmission of agglutinins of *Bacillus abortus* from cow to calf in the colostrum..... 161

HAMMETT, FREDERICK S., NOWREY, JOSEPH E., JR., and MÜLLER, JOHN H. The erythropoietic action of germanium dioxide.. 173

CHESNEY, ALAN M. The use of phenol red and brom-cresol purple as indicators in the bacteriological examination of stools..... 181

WARREN, S. L., and WHIPPLE, G. H. Roentgen ray intoxication. I. Unit dose over thorax negative—over abdomen lethal. Epithelium of small intestine sensitive to x-rays..... 187

WARREN, S. L., and WHIPPLE, G. H. Roentgen ray intoxication. II. A study of the sequence of clinical, anatomical, and histological changes following a unit dose of x-rays..... 203

WARREN, S. L., and WHIPPLE, G. H. Roentgen ray intoxication. III. Speed of autolysis of various body tissues after lethal x-ray exposures. The remarkable disturbance in the epithelium of the small intestine..... 213

MCQUARRIE, IRVINE, and WHIPPLE, G. H. A study of renal function in Roentgen ray intoxication. Resistance of renal epithelium to direct radiation..... 225

CUTLER, ELLIOTT C. The relation of the hypophysis to antibody production..... 243

DUVAL, CHARLES W., and D'AUNOY, RIGNEY. Studies upon experimental measles. I. The effects of the virus of measles upon the guinea pig..... 257

FENN, WALLACE O. Hemolysis of erythrocytes in contact with glass..... 271

No. 3, MARCH 1, 1922.

GRATIA, ANDRÉ. The Twort-d'Hérelle phenomenon. II. Lysis and microbic variation. Plates 7 and 8..... 287

KLIGLER, I. J., and ROBERTSON, O. H. The cultivation and biological characteristics of *Spirochæta obermeieri* (*recurrentis*) 303
LEWIS, MARGARET REED. The importance of dextrose in the medium of tissue cultures..... 317
STADIE, WILLIAM C. Construction of an oxygen chamber for the treatment of pneumonia. Plate 9..... 323
STADIE, WILLIAM C. The treatment of anoxemia in pneumonia in an oxygen chamber..... 337
JONES, F. S. An organism resembling *Bacillus actinoides* isolated from pneumonic lungs of white rats. Plate 10..... 361
FISCHER, ALBERT. A three months old strain of epithelium. Plates 11 to 14..... 367
MURPHY, JAMES B., LIU, J. HENG, and STURM, ERNEST. Studies on x-ray effects. IX. The action of serum from x-rayed animals on lymphoid cells *in vitro*. Plate 15..... 373
CLARK, HARRY. The measurement of intravenous temperatures. Plate 16..... 385
NOGUCHI, HIDEYO. Venereal spirochetosis in American rabbits. Plates 17 to 20..... 391

No. 4, APRIL 1, 1922.

ORCUTT, MARION L., and HOWE, PAUL E. Hemolytic action of a staphylococcus due to a fat-splitting enzyme..... 409
PAPPENHEIMER, A. M., MCCANN, G. F., and ZUCKER, T. F. Experimental rickets in rats. IV. The effect of varying the inorganic constituents of a rickets-producing diet. Plates 21 to 24..... 421
PAPPENHEIMER, A. M., MCCANN, G. F., and ZUCKER, T. F. Experimental rickets in rats. V. The effect of varying the organic constituents of a rickets-producing diet. Plates 25 to 29..... 447
BROWN, J. HOWARD. Modification of an improved anaerobe jar..... 467
OLITSKY, PETER K. Experimental studies on the etiology of typhus fever. IV. Immunizing and toxic agents found occasionally in filtrates of typhus-infected tissues. Plates 30 and 31..... 469

NAKAHARA, WARO, and MURPHY, JAMES B. Studies on x-ray effects. X. The biological action of small doses of low frequency x-rays. Plates 32 and 33..... 475
LIU, J. HENG, STURM, ERNEST, and MURPHY, JAMES B. Studies on x-ray effects. XI. The fate of cancer grafts implanted in subcutaneous tissue previously exposed to x-rays..... 487
NAKAHARA, WARO. Studies on lymphoid activity. VI. Immunity to transplanted cancer induced by injection of olive oil. Plates 34 to 36..... 493
HAMMETT, FREDERICK S., and NOWREY, JOSEPH E., JR. The erythropoietic action of germanium dioxide. II. The source of the erythrocythemia produced by germanium dioxide in the albino rat..... 507
HAESSLER, HERBERT. The effect of flood diuresis on hemoglobinuria..... 515
MCMASTER, PHILIP D., ROUS, PEYTON, and LARIMORE, LOUISE C. Significance of the hemosiderosis of pernicious anemia.. 521
HAESSLER, HERBERT, ROUS, PEYTON, and BROUN, G. O. The renal elimination of bilirubin..... 533
OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. VII. Serological reactions. Plate 37..... 553
DE KRUIF, PAUL H. Mutation of the bacillus of rabbit septicemia. Plate 38..... 561
DU NOÜY, P. LECOMTE. Spontaneous decrease of the surface tension of serum. I..... 575

No. 5, MAY 1, 1922.

SWIFT, HOMER F., BOOTS, RALPH H., and MILLER, C. PHILIP, JR. A cutaneous nematode infection in monkeys. Plates 39 to 52..... 599
DE KRUIF, PAUL H. Virulence and mutation of the bacillus of rabbit septicemia..... 621
GATES, FREDERICK L. Collodion sacs for aerobic and anaerobic bacterial cultivation. Plates 53 and 54..... 635
CARREL, ALEXIS, and EBELING, ALBERT H. Heat and growth-inhibiting action of serum..... 647

EBELING, ALBERT H. Cicatrization of wounds. XIII. The temperature coefficient. 657
FISCHER, ALBERT. Action of antigen on fibroblasts *in vitro* 661
BROWN, J. HOWARD. The vaseline tube and syringe method of micro gas analysis of bacterial cultures. Plates 55 to 57 . . . 667
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. I. Acid death-point of the pneumococcus. . 685
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. II. Dissolution of pneumococci at varying hydrogen ion concentrations. Effect of temperature, previous killing of the organisms, and fresh human serum on the phenomenon. Behavior of other organisms. 689
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. III. Dissolution of pneumococci in pneumonic cellular material at varying hydrogen ion concentrations. Resistance of certain other organisms to dissolution. . 699
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. IV. Effect of bile at varying hydrogen ion concentrations on dissolution of pneumococci. 703
DU NOÜY, P. LECOMTE. Surface tension of serum. II. Action of time on the surface tension of serum solutions. Plates 58 to 61. 707
FOOT, NATHAN CHANDLER. Pathology of the dermatitis caused by *Megalopyge opercularis*, a Texan caterpillar. Plates 62 and 63. 737

No. 6, JUNE 1, 1922.

EBELING, ALBERT H. A ten year old strain of fibroblasts. Plates 64 to 66. 755
FELTON, LLOYD D., and DOUGHERTY, KATHARINE M. Study of the action of four aromatic cinchona derivatives on pneumococcus. A comparison with optochin. 761
TYZZER, ERNEST EDWARD, and FABYAN, MARSHAL. A further inquiry into the source of the virus in blackhead of turkeys, together with observations on the administration of ipecac and of sulfur. 791

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. VIII. Further observations on the cultural and morphological characters of *Bacterium pneumosintes*. Plate 67..... 813
STEVENS, FRANKLIN A., and WEST, RANDOLPH. The peptase, lipase, and invertase of hemolytic streptococcus..... 823
KRUMBHAAR, EDWARD B., and CHANUTIN, ALFRED. Studies on experimental plethora in dogs and rabbits..... 847
INDEX TO VOLUME XXXV..... 873

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VI. Immunity Reactions.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 16, 1921.)

In the earlier experiments of this series in which the anaerobic, filter-passing microorganism, *Bacterium pneumosintes*, was transmitted from the nasopharyngeal secretions of influenza patients to the respiratory tract of rabbits, and passed from rabbit to rabbit through many generations, it was our practice to inoculate two or more rabbits in each experiment. One or more of these animals was killed after 24 to 48 hours, as already described,[1] for study and to obtain material for further inoculation. The infection, however, was permitted to run its course in a number of rabbits, which upon recovery became available for subsequent studies in immunity.

The experiments made with the latter rabbits may be grouped according to the nature of the injected material (Table I). These animals were reinjected after varying intervals with material from other sources. The tests were particularly useful in establishing the identity of the active agent derived from human nasopharyngeal washings, and from the lung tissues of affected rabbits, with the cultures of *Bacterium pneumosintes*.

A single typical protocol of the experiments will serve to show the method followed and the results obtained.

Group A. Influenzal Nasopharyngeal Washings versus Active Agent in Lungs of Affected Rabbits.

Experiment 1.—Rabbit A for 4 days prior to injection showed an average leucocyte count of 11,821, of which 6,190 were mononuclears, and temperature of 39.6°C. Mar. 29, 1919. Inoculated intratracheally with 3 cc. of the unfiltered nasopharyngeal washings from Case 16.[1] Mar. 30. Conjunctivitis. Total

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

leucocytes 9,000, of which 3,600 were mononuclears. Temperature 39.6°C. Mar. 31. Conjunctivitis. Total leucocytes 7,025, of which 1,967 were mononuclears. Temperature 40°C. Apr. 1. Conjunctivitis. Total leucocytes 6,325, of which 2,404 were mononuclears. Temperature 40°C. Apr. 2. Recovered. Total leucocytes 11,500, of which 4,140 were mononuclears. Temperature 39.4°C. The animal remained well until Apr. 21, 1919, when it was reinjected, intratracheally, with 3 cc. of lung tissue suspension from a rabbit representing the fifth passage of the nasopharyngeal secretions from Case 17.[1] This active material had been filtered between the third and fourth rabbit passages. No observable effects on the rabbit's appearance, behavior, blood count, or temperature resulted.

As a control for the first injection, Rabbit B was injected along with Rabbit A, and with the same material. For 2 days after the injection Rabbit B showed a typical clinical picture, with a fall in the leucocyte count, affecting especially the mononuclears, and a rise in temperature. The animal was then killed and autopsy revealed the characteristic hemorrhagic edema and emphysema of the lungs, without involvement of other organs.[1] Similarly, Rabbit C served as a control for the second injection of Rabbit A, and showed typical clinical and pathological effects.

TABLE I.

Groups of Immunity Experiments.

Group.	First injection.	Second injection.
A	Nasopharyngeal washings from influenza patients.	Suspensions of lungs of affected rabbits.
B	Suspensions of lungs of affected rabbits.	Similar suspensions from another animal.
C	Washed mass cultures of *Bacterium pneumosintes.*	Similar cultures of another strain.
D	Suspensions of lungs of affected rabbits.	Washed mass cultures of *Bacterium pneumosintes.*
E	Washed mass cultures of *Bacterium pneumosintes.*	Suspensions of lungs of affected rabbits.

This experiment, typical of three in this series, indicates the development of a protective resistance to the active agent in the rabbit passage experiments, as the result of the previous injection of the nasopharyngeal secretions from an early case of influenza. The nasopharyngeal secretions are thus further shown to be the source of the filterable active material of the rabbit passages. In this instance,

the interval between injections was 23 days. In another experiment, the resistance was maintained for 3 months.

Group B. Active Agent in Lungs of Affected Rabbits versus Similar Material Originally Derived from Other Patients.

That the strains of the active agent in lungs of affected rabbits, originally derived from different influenza patients, have the same antigenic properties and thus give rise to protection against each other is brought out by the following experiment.

Experiment 2.—Rabbit A, having a normal leucocyte count of 14,525, of which 6,100 were mononuclears, and a temperature of 39.8°C., was injected intratracheally on Feb. 25, 1919, with 3 cc. of a saline solution suspension of rabbit lung from the fourth passage of the nasopharyngeal secretions of Case 11,[1] obtained during the first influenzal wave of 1918–19. The subsequent train of events carried the mononuclear cells to 1,242, and the temperature to 40.65°C. on the 3rd day. The fever and mononuclear leucopenia persisted on the 4th and 5th days, with recovery on the 6th and 7th days, when the total leucocyte count was 20,450, of which 5,521 were mononuclears, and temperature 39.6°C. Nearly 14 months later, May 11, 1920, this animal was injected with 3 cc. of a suspension of glycerolated lung tissue from the first rabbit inoculated with the nasopharyngeal secretions of Case 26,[1] from the second or 1920 epidemic. The rabbit developed no significant changes in the blood count or temperature, and when killed, on May 13, showed no visible lung lesions.

The control rabbit, B, similarly injected with the same glycerolated lung tissue, developed the typical blood picture and lung lesions, and the presence of the active agent in the lungs was demonstrated by transmission to a second rabbit which reacted in the characteristic manner.

This experiment not only demonstrates the identity of the antigenic characters of the active material derived from the two epidemics of 1918–19 and of 1920, but shows that the immunity conferred by a previous infection with the active agent may last for a long time.

With the isolation of *Bacterium pneumosintes* from the nasopharyngeal secretions of influenza patients and from the lung tissue of affected rabbits, it became possible to study the character of the active agent of the animal passages by means of cross-protection experiments. It was first necessary to show that recovery from infection with *Bacterium pneumosintes* induces resistance to a subsequent inoculation of the same microorganism.

Group C. Bacterium pneumosintes versus the Same Microorganism.

Experiment 3.—Rabbit A, normal leucocyte count 9,600, of which 6,144 were mononuclears. Temperature 39.3°C. Injected intratracheally with 3 cc. of a saline suspension of washed mass culture in the seventh generation of a rabbit lung strain of *Bacterium pneumosintes* from Case 16,[1] a patient from the first (1918–19) epidemic wave. 1st day after injection: total leucocytes 8,400, of which 2,520 were mononuclears; temperature 39.3°C. 2nd day: total leucocytes 3,800, of which 1,444 were mononuclears; temperature 39.6°C. 3rd day: total leucocytes 5,400, of which 2,052 were mononuclears; temperature 39.6°C. 4th day: recovery; total leucocytes 11,600, of which 4,640 were mononuclears; temperature 39.3°C. 5th day: total leucocytes 15,000, of which 7,500 were mononuclears; temperature 39.4°C. On the following day, when the total leucocytes were 13,600, of which 5,440 were mononuclears, and the temperature was 39.8°C., this animal was reinjected with a similar washed mass culture of a strain of *Bacterium pneumosintes* in the seventh generation from the filtered nasopharyngeal secretions of Case 17,[1] from the same epidemic. During the next 5 days the total leucocyte count remained about 13,000, with an increase in the mononuclear cells to an average of 8,292, and the temperature was constant at 39.7°C.

For assurance that the injected mass culture was active and capable of infecting normal rabbits, a control animal was injected, each time, along with Rabbit A, and with the same material. For 48 hours after injection, these rabbits, B and C, showed the characteristic leucocytic depression, involving especially the mononuclears. In each instance they were then killed, and the usual emphysema and hemorrhagic edema of the lungs were found.[2]

To show that the reactions described were due to *Bacterium pneumosintes* and not to some constituent of the culture medium, Rabbit D was inoculated intratracheally with 3 cc. of a saline suspension of the sediment of uninoculated, sterile, mass culture medium, at the time that Rabbit A was first injected. The animal gave no reaction. At the time of the second injection, Rabbit D was also injected with the seventeenth generation of the culture of the nasopharyngeal secretions of Case 17. This animal promptly developed the clinical and pathological syndrome characteristic of infection with *Bacterium pneumosintes*.[2]

Group D. Active Agent in Lungs of Affected Rabbits versus Bacterium pneumosintes.

We have already stated[2] that "the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism" (*Bacterium pneumosintes*).

[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

Additional evidence in support of this statement is furnished by the results of cross-protection experiments of which the following are examples.

Experiment 4.—Rabbit A, normal leucocyte count, 9,200, of which 6,072 were mononuclears. Temperature 39°C. Injected intratracheally with 3 cc. of a saline solution suspension of rabbit lung tissue representing the fifth passage of the filtered nasopharyngeal secretions from Case 26.[1] The following day, leucocytes were 6,400, of which 2,560 were mononuclears. Temperature 39.3°C. 2nd day: leucocytes 7,200, of which 2,763 were mononuclears; temperature 39.4°C. 3rd day: leucocytes 4,400, of which 1,760 were mononuclears; temperature 39.4°C. 4th day: recovery with leucocytes 11,000, of which 6,600 were mononuclears; temperature 39.2°C. 15 days later this rabbit was injected intratracheally with 3 cc. of a saline solution suspension of washed mass culture of *Bacterium pneumosintes*, second generation, originally derived from the nasopharyngeal secretions of the same patient. On the 2 days following, the total leucocyte counts were 14,600 and 16,800, of which 7,800 and 9,240 were mononuclears; temperature 39.5°C. Killed. Autopsy revealed no visible lesions in the lungs. The activity of the materials used in these injections was demonstrated by the injection of control rabbits, B and C, both of which gave typical reactions. In similar experiments, the reinjected rabbits were observed for several days after the second injection to detect any delayed effects. They remained normal.

In Experiment 4, the interval between the first and second injections was 15 days. In three other experiments, identical results were obtained after 3 weeks, and in a fifth, after 9 weeks. In a sixth experiment, involving an interval of 18 days, the second injection produced the typical clinical and pathological reaction. That such protection experiments should occasionally be unsuccessful is not surprising, in view of the arbitrary dosage employed.

Group E. Bacterium pneumosintes versus Active Agent in Lungs of Affected Rabbits.

The converse of Experiment 4 was performed in a series in which *Bacterium pneumosintes* was injected first and the animals were later exposed to the influenzal active agent by the intratracheal injection of affected rabbit lung.

Experiment 5.—Rabbit A showed a normal leucocyte count of 12,000, of which 6,240 were mononuclears. Temperature 39°C. It was injected intratracheally with 3 cc. of a saline solution suspension of washed sediment from a mass culture

of *Bacterium pneumosintes*. During the following 3 days, the total leucocyte count averaged 7,200, of which 2,200 were mononuclears, and the temperature rose to 39.6°C. Thereafter the animal returned to normal. 2 weeks after the first injection, this rabbit was inoculated intratracheally with 3 cc. of a suspension of the active agent in rabbit lung tissue from the fifth passage of the nasopharyngeal secretions of Case 26.[1] After waiting for 48 hours, during which time no change occurred in the blood or temperature, the rabbit was killed. The lungs appeared normal.

In three other similar experiments the rabbits were observed for several days after the second injection, and remained normal. The interval between injections in this series was 11 days, 2, 7, and 8 weeks. The usual control animals, B and C, demonstrated the activity of the injected materials in each test.

These cross-protection experiments establish the antigenic identity of *Bacterium pneumosintes* with the active agent derived from the nasopharyngeal secretions of patients in the early hours of epidemic influenza.

SUMMARY AND CONCLUSION.

The experiments described furnish additional evidence of the pathogenic character and the virtual identity of the various strains of the active agent derived from the nasopharyngeal secretions of influenzal patients with which the transmission experiments in rabbits have been carried out.

The active material has been shown to be of antigenic nature, so that rabbits are protected from the effects of a second inoculation. The experiments indicate also the antigenic identity of the various strains of the active agent with each other and with *Bacterium pneumosintes*.

Finally, the experiments show that the protection may persist for 14 months which is the longest period yet tested.

THE FREQUENCY OF BACILLUS INFLUENZÆ IN THE NOSE AND THROAT IN ACUTE LOBAR PNEUMONIA.

By ERNEST G. STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 15, 1921.)

The writer has for some time been engaged in the study of the flora of the nose and throat both in well persons and in those suffering from infections of the respiratory tract. Especial attention has been given to the presence of *Bacillus influenzæ* and pneumococcus, and some of the results obtained have already been published (1).

During the course of these studies, certain observations have been made concerning the frequency of occurrence of influenza bacilli and pneumococci in the nose and throat, and concerning the varieties of influenza bacilli which are present in these regions. It is possible that these findings may have a bearing upon the etiology of lobar pneumonia, and for this reason they are reported here.

Methods.

The throat cultures were made by means of cotton swabs passed over the posterior wall of the pharynx. Two swabs were used in each case—with one a freshly poured oleate hemoglobin agar plate was inoculated, with the other a fresh blood agar plate.

Cultures from the nose were made by means of a cotton swab passed through a sterile speculum in order to avoid contamination from the nasal hairs. Cultures were usually made from the nasal mucous membrane in the region of the middle turbinate, though occasionally a deviated septum prevented the introduction of the swab as far as this region. The swabs were streaked over freshly poured blood agar plates.

Attention was given not only to the occurrence of colonies of pneumococcus and of *Bacillus influenzæ* on the various plates, but an

effort was made to determine the type of influenza bacilli present and especially whether bacilli of more than one type could be isolated from a single culture. For this reason, whenever colonies of *Bacillus influenzæ* were present, several individual colonies were marked and a secondary culture on a fresh blood agar slant was made from each of the colonies. In the present paper, the occurrence of hemoglobinophilic bacilli producing hemolysis on blood agar plates, the so called Bacillus X, is not discussed and the presence of this organism is not noted. All organisms here described as *Bacillus influenzæ* failed to produce hemolysis.

The media used in this study had an initial hydrogen ion concentration of about pH 7.4. The blood agar was prepared by adding sterile defibrinated rabbit blood to melted agar to a concentration of 4 per cent or by adding blood extract, according to Wollstein's (2) method, to a concentration of 2 per cent.

In this connection, it should be mentioned that all solid media employed in the cultivation of *Bacillus influenzæ* should be freshly prepared. The addition of a little broth to the surface of old blood agar plates will not give satisfactory results. In the present study, the plates were poured within an hour of use. For making fresh oleate hemoglobin plates, it is well to employ tubes of oleate medium which may be melted as needed. Care must be taken that the blood cells are completely hemolyzed before the oleate agar is tubed. If this is not done, upon remelting the agar a heavy protein precipitate will be formed.

Indole production was tested by Ehrlich's para-dimethylaminobenzaldehyde method. The clear supernatant fluid from 48 hour cultures in blood broth or in Dunham's peptone solution enriched with blood extract was used. Since an occasional strain, which apparently had grown luxuriantly, failed to produce indole on the first test, all tests resulting negatively were repeated at least once.

Gas production was tested by making either stab or shake cultures in 1 per cent dextrose agar to which blood extract had been added. Since the amount of gas produced is never very great, all negative tests were repeated.

The sugar fermentation tests were made with cultures in Dunham's peptone solution enriched with 2 per cent blood extract. All cul-

ture tubes were incubated in a slanted position so as to expose as large a surface of the medium as possible to the air, since acid production has been shown to be more rapid under these conditions. As a routine procedure, dextrose, which 92 per cent of the strains fermented, was used as a control, and saccharose as the differential sugar.

Occurrence of Bacillus influenzæ in the Upper Respiratory Tract of Normal Individuals and of Individuals Suffering from Lobar Pneumonia.

During the recent epidemic of influenza in New York in 1918–19 and in the succeeding winters, the writer made many cultures from the throats of normal individuals to determine the presence or absence of *Bacillus influenzæ*. During the period 1918–21, cultures were made from the throats of 1,077 normal individuals, and influenza bacilli were demonstrated to be present in 332, or in 30 per cent (Table I).

While cultures have been made from the nose in a much smaller number of normal individuals and then only during the past year, 1920–21, the results indicate that hemoglobinophilic bacilli are rarely found here, even in persons suffering from coryza, laryngitis, etc. (Table II).

In marked contrast with these findings are the results obtained in a series of cultures made from the nose and throat of patients suffering from lobar pneumonia. In thirty-one of these cases, cultures were made from the throat, and in eighteen, or from 58 per cent of the cases, influenza bacilli were isolated (Table I). In thirty-five cases of pneumonia, cultures were also made from the nose and influenza bacilli were isolated from this source in nine, or in 23 per cent (Table II). In certain other cases influenza bacilli were isolated from the sputum when they could not be demonstrated in the cultures from the nose or throat. Among the entire thirty-five cases, influenza bacilli were isolated from at least one of these three sources in thirty, or 85 per cent of the cases. These cultures were made during the winter of 1920–21, 2 years after the severe epidemic of influenza. During this winter few cases of typical influenza occurred in New York. That this great frequency of association of *Bacillus influenzæ* with patients suffering from pneumonia

is not directly related to the epidemic is further rendered probable by the fact that during the 5 years previous to the epidemic, namely from 1913–18, influenza bacilli were isolated from the sputum of 20 per cent of 672 cases of pneumonia studied in the Hospital of The Rockefeller Institute. While the relative number of instances in which influenza bacilli were isolated from this series of pneumonia

TABLE I.

Incidence of B. influenzæ in Throat Cultures from Normal Individuals and from Individuals Suffering from Lobar Pneumonia.

Date.	Total No. of individuals.	Positive cultures.	Per cent positive.
Normal individuals.			
Winter of 1918–19	717	256	35
" " 1919–20	253	44	17
" " 1920–21	107	32	29
Total	1,077	332	30
Patients with lobar pneumonia.			
1920–21	31	18	58

TABLE II.

Incidence of B. influenzæ in the Nose of Normal Individuals and of Individuals Suffering from Acute Coryza, Laryngitis, Encephalitis Lethargica, and Lobar Pneumonia.

Source of culture.	No. of cases.	Cultures.	*B. influenzæ* present.
Normal individuals	22	28	—
Acute coryza and laryngitis	13	23	—
Encephalitis lethargica	2	2	—
Lobar pneumonia	35	35	9

patients is not as great as the percentage of normal persons who in 1918–21 were found to be carrying these organisms, it must be borne in mind that influenza bacilli were only incidentally looked for in the pneumonia patients previous to 1918 and were only brought to our attention by their growth in the mouse into which sputum had been injected. These figures indicate that influenza bacilli

are much more frequently present in the upper respiratory tract of patients with lobar pneumonia than of normal persons, or at any rate can be more frequently isolated. This is especially true of the occurrence of these bacilli in the throat but is also true of their occurrence in the nose.

In searching for influenza bacilli in the nose, we have also noted the presence or absence of pneumococcus. In the cultures from the nose of the twenty-two normal individuals mentioned in Table II, it was possible to isolate pneumococcus in only one instance. More surprising were the results obtained from nasal cultures from the thirteen cases of coryza and laryngitis and from the two cases of encephalitis lethargica. In these instances no pneumococci were found. Park (3), Allen (4), and others have previously shown that cultures from the nose of normal individuals usually yield a very scanty growth of bacteria. Not infrequently cultures from this region have been found to be sterile.

On the other hand, we have made cultures from the nose in thirty-five cases of lobar pneumonia, and in fifteen instances pneumococci were demonstrated to be present. In the majority of instances, the type of pneumococcus isolated was determined and the organism was found to be identical with those associated with the lung lesion as shown by the sputum determination.

Types of Bacillus influenzæ in Nose, Sputum, and Throat Cultures from Individuals Suffering from Lobar Pneumonia.

In the classification of the influenza bacilli, use was made of the grouping devised by Stillman and Bourn (5). The following chart indicates briefly the method employed and the mode of identifying the various groups.

	Group.					
	A.	B.	C.	D.	E.	F.
Indole	+	−	−	−	+	−
Gas	−	+	−	−	−	+
Saccharose	−	+	+	−	+	−

From a study of the hemoglobinophilic bacilli isolated, it soon became evident that the organisms present in any one case or even in any one culture were not all of the same type. It was therefore impossible to group the cases according to the presence of *Bacillus*

influenzæ of the different types or even of the prevailing type. A study was made, therefore, of all the strains isolated from cases of lobar pneumonia during the winter of 1920–21 and the results are shown in Table III.

It is evident from this table that the strains of *Bacillus influenzæ* isolated from the nose, throat, and sputum of patients with lobar pneumonia do not belong in any one group, but that representatives of all groups occur, though the strains belonging in Group A are in the majority.

Of still greater importance than this, however, is the fact that in certain instances the study of several colonies from the same culture

TABLE III.

A Biological Classification of 140 Strains of B. influenzæ Recovered from the Throat, Sputum, and Nose of Thirty-five Individuals Suffering from Lobar Pneumonia.

Source of culture.	Total No. of cultures.	No. of strains studied.	Group.					
			A.	B.	C.	D.	E.	F.
Throat	32	91	32	18	7	32	2	—
Sputum *via* mouse	8	18	8	—	2	8	—	—
Nasal	35	31	18	2	1	10	—	—
Total	75	140	58	20	10	50	2	—

B. influenzæ present in 30 cases.
" " absent " 5 "
Total 35 "

plate from the nose or throat has indicated that influenza bacilli of several different groups may be simultaneously present. For instance, in one case from a single plate made from the throat, of five colonies of *Bacillus influenzæ* studied, two colonies were shown to consist of influenza bacilli of Group C, and three of influenza bacilli of Group D, and in another case three colonies from a throat culture plate were studied and the bacilli from each of the colonies were found to belong in a different group.

Of seven cases of lobar pneumonia in which both nose and throat cultures yielded *Bacillus influenzæ*, in three a single type of organism was recovered from both nose and throat. In four instances, different type strains were recovered from these two sources.

DISCUSSION.

At the present time, the significance of the above findings is not definite, though certain explanations suggest themselves. Evidence has been accumulating which indicates that the mere presence of pathogenic pneumococci in the upper respiratory tract, or even in the lungs, is in the great majority of cases, possibly in all, not sufficient in itself to initiate the disease lobar pneumonia in man. It is true that Blake and Cecil (6) have shown that pneumonia may be induced in monkeys merely by the introduction of virulent pneumococci into the trachea, but the amount of culture employed was of considerable size, and in any case, in natural infection in man some preceding event would have to be postulated which would make it possible for the organisms to reach the trachea in sufficient numbers to initiate the disease. Furthermore, Blake and Cecil found it difficult to transmit Type I pneumonia from monkey to monkey by intimate and prolonged contact alone, only one out of six attempts being successful. They were also unable to initiate pneumonia in monkeys by the instillation of large amounts of a culture of virulent pneumococcus into the nose and throat, though the monkeys carried pneumococcus in the mouth for a month. From these observations, it seems likely that the mere presence of pneumococci in the normal upper respiratory tract, or even in the lung, can rarely, if ever, be held accountable for the onset of pneumonia.

More and more attention is therefore being given to the factors which are concerned in etiology previous to actual infection by the pneumococcus. It has been shown that 40 per cent of the cases of lobar pneumonia give a history of coryza (7) or other mild infection of the respiratory tract preceding the onset of pneumonia. It seems quite possible that a variety of contributing factors at present unknown, such as mild unrecognized infections, may act in lowering the local or general bodily resistance so that the pneumococcus may invade and infect the lungs.

Our observation that *Bacillus influenzæ* and pneumococci are frequently present in the nose and throat of pneumonia cases therefore suggests that these organisms may have been responsible for a coryza or infection of the mucous membranes of the nasopharynx preliminary

to the infection of the lung with the pneumococcus. It must be borne in mind, however, that the relation of these organisms to ordinary colds is at present purely hypothetical. In any case, our observations indicate that in coryza and laryngitis, it is unusual for these organisms to be present in the nose.

Another possible explanation is that during the course of acute lobar pneumonia, the entire mucous membrane of the respiratory tract loses certain properties antagonistic to the growth of microorganisms, and that under these conditions all varieties of bacteria which normally grow sporadically are able to grow with great vigor. At present, however, the exact significance of our observation cannot be established and the above possibilities are offered merely as suggestions.

The fact that the hemoglobinophilic bacilli found in the upper respiratory tract of a given individual under the conditions discussed are not all of the same variety, is at first confusing and difficult to understand. One should bear in mind, however, the conditions in regard to the pneumococcus about which knowledge has been greatly increased during recent years. It is well known that pneumococci are of several types. Epidemiological evidence indicates that the Type IV pneumococci are relatively harmless and are normal inhabitants of the upper respiratory tract, while Type I and Type II pneumococci are almost always found in association with disease. It is not infrequent to find two types of pneumococci in one mouth, especially in the mouths of persons who have been in close association with pneumonia patients, but up to the present time the writer has never encountered a Type I and a Type II pneumococcus in the mouth at the same time. When pneumococci of one of these two types are present, together with pneumococci of Type IV, it is probable that the latter were present before the advent of the Type I or Type II pneumococci. Furthermore, the work of Olmstead (8) indicates that Type IV pneumococci may be further subdivided into definite types. Whether all the pneumococci of Type IV which are found in a single mouth are identical or not is not certain. It is not at all impossible that they may be of different kinds.

As regards hemoglobinophilic bacilli, the methods which have so far been proposed for separating them into groups or types cannot be said to be satisfactory. It is not possible at present to separate the saprophytic from the disease-producing varieties. The tentative classification based on indole production, gas formation, and saccharose fermentation which we have proposed may not be based on fundamental differences in the bacteria. When further knowledge concerning this group is obtained, the presence of several varieties in one mouth may possibly be shown to depend upon conditions analogous to those relating to pneumococcus.

SUMMARY.

1. *Bacillus influenzæ* is frequently found in association with lobar pneumonia.

2. In lobar pneumonia both *Bacillus influenzæ* and pneumococcus are frequently found in the nose.

3. The influenza bacilli found in the upper respiratory tract of cases of lobar pneumonia are of various types.

4. The exact significance of these findings is at the present time not clear.

BIBLIOGRAPHY.

1. Stillman, E. G., *J. Exp. Med.*, 1916, xxiv, 651; 1917, xxvi, 513. Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259. Winchell, A. I., and Stillman, E. G., *J. Exp. Med.*, 1919, xxx, 497.
2. Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445. Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxxi, 2050.
3. Park, W. H., and Wright, J., *J. Laryngol., Rhinol. and Otol.*, 1898, xiii, 124.
4. Allen, R. W., *Lancet*, 1908, ii, 1589.
5. Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665.
6. Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.
7. Cole, R., in Nelson, Loose-leaf medicine. A perpetual system of living medicine, New York, 1920, i, 203.
8. Olmstead, M., *J. Immunol.*, 1916–17, ii, 425.

HETEROGENIC SERUM, AGE, AND MULTIPLICATION OF FIBROBLASTS.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 21, 1921.)

I.

INTRODUCTION.

It has been shown that the inhibiting action of homogenic serum on the growth of a pure culture of chicken fibroblasts is directly proportional to the age of the animal from which the serum is taken, and that the rate of cell multiplication can be used as a reagent of certain changes produced by age in the blood serum.[1] Similar effects probably could be obtained from fibroblasts of mammals cultivated in homogenic serum. But it would be of advantage to cultivate chicken tissue in heterogenic serum because it is much more difficult to keep alive *in vitro* permanently a strain of mammal fibroblasts than of chicken fibroblasts. Over 10 years ago we found that chick embryo tissues grew abundantly in plasma from rabbits, dogs, and human beings, although not quite as well as in chicken plasma.[2] Later the effect of many heterogenic sera was investigated. Lambert and Hanes[3] cultivated rat sarcoma in guinea pig plasma and Ingebrigtsen[4] studied the action of several heterogenic sera on cultures of guinea pig bone marrow. The growth was always less rapid and extensive than in homogenic serum, and the toxic action of the serum varied according to the species of the animal. Owing to the lack of accuracy of the technique used at the time, no precise knowledge was gained of the action of these different sera.

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.
[2] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiv, 244.
[3] Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiv, 129.
[4] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397; xvi, 421.

A quantitative study of the influence of heterogenic sera, taken from animals of different ages, on the rate of growth of pure cultures of fibroblasts was begun by one of us in 1919. Fragments of a 7 year old strain of chicken fibroblasts were cultivated in media made of one volume of plasma and two volumes of serum taken from cats 1 month, 1 year, and 9 years old. The amount of growth obtained in the kitten serum was about four times greater than in the old cat serum. Similar experiments were made with serum obtained from human beings 22, 40, and 45 years old. Differences in the rate of growth were observed, although they were less marked than in cat serum. It became evident that the growth of chicken fibroblasts was more active in the serum of the young than in that of the old animals. When the technical improvements recently described by Ebeling rendered it possible to cultivate tissues in a medium composed of serum and fibrinogen,[5] and to prepare the cultures and measure the rate of growth with greater accuracy,[6] the study of the action of heterogenic serum was resumed. The purpose of the experiments described in this paper was to investigate the relation existing between the rate of growth of a pure culture of fibroblasts and the concentration in the medium of heterogenic serum, and the influence of the age of the animals from which the heterogenic serum was taken on the cell multiplication, in order to ascertain whether a pure culture of chicken fibroblasts could be used as a reagent for the detection of the modifications brought about by age in the blood of mammals.

II.

Action of Various Concentrations of Heterogenic Serum.

The action of different dilutions of homogenic and heterogenic sera on the rate of growth of chicken fibroblasts was thus investigated. The fibroblasts were taken from stock cultures of a 9 year old strain of chicken connective tissue, cultivated in one volume of embryonic tissue juice to two volumes of chicken plasma. Each fragment was divided in two equal parts, one being placed in the experimental medium and the other in the control. The experimental

[5] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.
[6] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

medium was composed of 20 per cent fibrinogen suspension, 5 per cent embryo juice, and varied amounts of Tyrode solution and dog or cat serum. The control medium had a similar composition, but the heterogenic serum was replaced by chicken serum. The fibrinogen suspension was prepared according to the method described by Ebeling.[5] The embryo juice was obtained from 8 or 9 day old chick embryos. Serum was taken from the plasma of cats or dogs under 2 years of age.

The action of media containing from 5 to 65 per cent heterogenic serum was compared with that of media containing identical amounts of chicken serum. Three cultures were made in each concentration of heterogenic serum. They were controlled by three cultures made in similar concentrations of chicken serum. The results of the experiments, showing the action of dog serum, were expressed by the relative increase of the tissues in 48 hours and are recorded in Tables I and II. The three experiments and controls of each series were averaged and the ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, was calculated. The variations in the rate of growth were expressed by a graph in which the ratio was plotted in ordinates and the serum concentration in abscissæ (Text-figs. 1 and 2). The rate of growth did not differ markedly from the control as long as the concentration of heterogenic sera was lower than 20 per cent. When the concentration of dog serum was increased from 20 to 35 per cent, the inhibiting action of the medium rapidly became more marked, and no growth was observed when the concentration reached 35 to 40 per cent. Other experiments showed that according to the individual, the serum concentration which prevents growth may vary from 30 to 45 per cent. The ratio of both relative increases in dog and chicken sera expressed the inhibiting action of dog serum on the proliferation of the fibroblasts, and can be called growth index of the serum. The growth index of the dog sera for a concentration of 30 per cent was respectively 0.32 and 0.067 (Tables I and II). The growth index of the cat sera for the same concentration (30 per cent) was 0.83 and 0.84. In other experiments, the growth index was also calculated in comparing the relative increases of a pure culture of fibroblasts in dog serum and in Tyrode solution, instead of chicken

TABLE I.

Experiment 1. Influence of Various Concentrations of Dog and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																	
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.	
		Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.
1	21864	4.25	4.9														
2	21865	6.5	6.0														
3	21866	6.3	6.06														
4	21799			4.42	2.78												
5	21800			5.54	2.52												
6	21801			3.8	2.46												
7	21880			4.8	4.2												
8	21881			4.7	4.67												
9	21882			5.6	4.9												
10	21930					3.6	3.5										
11	21931					3.5	2.9										
12	21932					3.8	3.3										
13	21769							2.33	3.04								
14	21928							4.2	3.05								
15	21929							3.4	2.2								
16	21950							3.7	3.5								
17	21951							3.16	2.65								

18	21912									3.0	2.1						
19	21913									3.3	2.3						
20	21914									3.6	1.7						
21	21802											5.0	2.95				
22	21803											3.7	0.95				
23	21804											4.23	0.51				
24	21861													3.3	0		
25	21862													3.58	0		
26	21863													3.6	0		
27	21770															2.7	0
28	21910															5.5	0
29	21911															3.7	0
Average		5.68	5.65	4.81	3.59	3.63	3.2	3.36	2.88	3.3	2.0	4.31	1.47	3.49	0	3.96	0
Relative increase in dog serum		0.96		0.75		0.89		0.93		0.62		0.32		0		0	
Relative increase in chicken serum																	

TABLE II.

Experiment 2. Influence of Various Concentrations of Dog and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.													
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.	
		Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.
1	21975	6.5	7.06												
2	21976	4.9	4.5												
3	21977	5.6	4.8												
4	21978			5.4	5.4										
5	21979			4.8	4.3										
6	21980			4.5	4.4										
7	21993					2.5	2.1								
8	21994					3.7	3.5								
9	21995					3.6	3.7								
10	21996							3.2	2.8						
11	21997							3.0	2.2						
12	21998							3.5	2.3						
13	22012									3.2	1.3				
14	22013									3.7	1.1				
15	22014									3.3	1.3				

16	22015											3.8	0.23		
17	22016											3.0	0.25		
18	22017											3.4	0.21		
19	22036													4.1	0.06
20	22037													3.6	0
21	22038													3.45	0
Average		5.66	5.45	4.9	4.7	3.26	3.1	3.2	2.43	3.4	1.2	3.4	0.23	3.7	0.02
Relative increase in dog serum		0.96		0.96		0.98		0.75		0.36		0.067		0.005	
Relative increase in chicken serum															

TABLE III.

Experiment 1. Influence of Various Concentrations of Cat and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.													
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.	
		Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.
1	22052	5.70	5.20												
2	22053	7.80	6.50												
3	22054	5.50	4.90												
4	22055			5.00	4.90										
5	22056			4.70	4.70										
6	22057			5.60	5.50										
7	22063					4.26	4.00								
8	22064					4.70	4.56								
9	22065					4.50	4.70								
10	22066							4.35	4.80						
11	22067							5.05	5.20						
12	22068							5.50	5.40						
13	22084									3.68	3.65				
14	22085									4.19	4.00				
15	22086									4.20	4.14				

16	22087											4.30	3.70		
17	22088											3.93	2.86		
18	22089											3.26	3.00		
19	22153													3.15	2.38
20	22154													3.77	2.40
21	22155													3.73	2.53
Average		6.33	5.53	5.10	5.03	4.49	4.42	4.97	5.13	4.02	3.93	3.83	3.19	3.55	2.44
Relative increase in cat serum		0.87		0.99		0.98		1.03		0.98		0.83		0.69	
Relative increase in chicken serum															

* The results obtained in higher dilutions were not recorded on account of errors due to liquefaction of the medium.

TABLE IV.

Experiment 2. Influence of Various Concentrations of Cat and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase. Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.		Serum concentration 50 per cent.		Serum concentration 55 per cent.		Serum concentration 60 per cent.	
		Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.
1	22242–1 & 2	5.58	5.06																						
2	22243–1 & 2	4.56	4.69																						
3	22244–1 & 2	4.72	4.87																						
4	22245–1 & 2			4.90	4.70																				
5	22246–1 & 2			3.86	4.90																				
6	22247–1 & 2			4.56	4.44																				
7	22251–1 & 2					3.65	4.24																		
8	22252–1 & 2					3.96	4.24																		
9	22253–1 & 2					3.74	2.13																		
10	22254							4.09	4.26																
11	22255							4.30	3.18																
12	22256							3.55	2.83																
13	22257									3.57	3.02														
14	22258									3.57	3.24														
15	22259									3.00	2.83														
16	22266											3.85	3.18												
17	22267											4.14	3.26												
18	22268											3.84	3.48												

19	22269													4.27	2.80									
20	22270													3.91	3.25									
21	22271													4.09	3.00									
22	22272															3.60	2.17							
23	22273															3.03	2.08							
24	22274															4.18	2.39							
25	22287																	3.60	1.73					
26	22288																	3.65	1.71					
27	22289																	4.00	1.47					
28	22290																			3.75	0.83			
29	22291																			3.56	0.91			
30	22292																			3.44	1.13			
31	22310																					4.45	0.47	
32	22311																					3.50	0.19	
33	22312																					4.23	0.25	
34	22313																							4.05
35	22314																							3.13
36	22315																							4.76
Average		4.95	4.87	4.44	4.68	3.78	3.54	3.98	3.42	3.38	3.03	3.94	3.30	4.09	3.02	3.60	2.21	3.75	1.64	3.58	0.96	4.06	0.30	3.98
Relative increase in cat serum / Relative increase in chicken serum		0.99		1.06		0.94		0.86		0.89		0.84		0.74		0.61		0.44		0.27		0.074		0.023

19	22269
20	22270
21	22271
22	22272
23	22273
24	22274
25	22287
26	22288
27	22289
28	22290
29	22291
30	22292
31	22310
32	22311
33	22312
34	22313
35	22314
36	22315

Average	0.09
Relative increase in cat serum / Relative increase in chicken serum	

19	
20	
21	
22	
23	
24	
25	
26	
27	
28	
29	
30	
31	
32	
33	
34	No growth.
35	0.14
36	0.14

TABLE V.

Influence of Various Concentrations of Young and Old Cat Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																			
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.		Serum concentration 50 per cent.		Serum concentration 55 per cent.	
		Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.
1	22299–1 & 2	5.30	4.83																				
2	22300–1 & 2	4.06	3.88																				
3	22301–1 & 2	3.61	3.47																				
4	22302–1 & 2			4.70	4.08																		
5	22303–1 & 2			4.33	4.58																		
6	22304–1 & 2			4.80	4.60																		
7	22359–1 & 2					5.47	4.70																
8	22360–1 & 2					4.35	3.88																
9	22361–1 & 2					4.75	4.21																
10	22362–1 & 2							4.32	4.67														
11	22363–1 & 2							5.02	4.56														
12	22364–1 & 2							5.12	4.06														
13	22365–1 & 2									4.93	4.65												
14	22366–1 & 2									4.80	4.12												
15	22367–1 & 2									4.15	2.98												
16	22382–1 & 2											3.52	2.94										
17	22383–1 & 2											3.66	2.98										
18	22384–1 & 2											3.10	2.56										

19	22385–1 & 2													4.00	2.90								
20	22386–1 & 2													3.30	2.23								
21	22387–1 & 2													2.35	2.00								
22	22388–1 & 2															2.37	1.91						
23	22389–1 & 2															2.32	1.15						
24	22390–1 & 2															2.76	1.19						
25	22443																	2.67	1.32				
26	22444																	2.40	1.00				
27	22445																	2.50	1.10				
28	22446																			2.10	0.64		
29	22447																			2.24	0.70		
30	22448																			2.11	0.73		
31	22449																					1.30	0
32	22450																					1.50	0
33	22451																					1.25	Few cells.
Average		4.32	4.06	4.61	4.42	4.85	4.26	4.82	4.43	4.62	3.91	3.43	2.83	3.22	2.38	2.48	1.42	2.52	1.14	2.15	0.69	1.35	0
Relative increase in old serum		0.94		0.96		0.91		0.92		0.84		0.82		0.74		0.57		0.45		0.32		0	
Relative increase in young serum																							

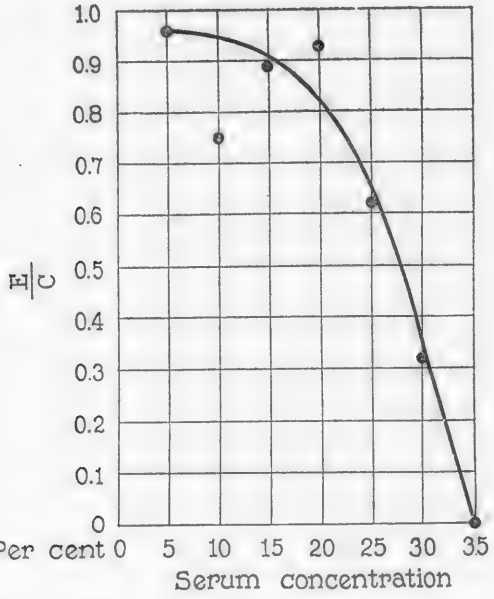
TABLE VI.

Influence of Various Concentrations of Young and Old Dog Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																	
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.	
		Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.
1	22598	4.48	4.13																
2	22599	4.73	4.56																
3	22600	3.90	3.36																
4	22601			3.21	3.19														
5	22602			3.80	3.75														
6	22603			3.88	4.18														
7	22604					3.40	3.16												
8	22605					2.88	2.88												
9	22606					2.74	2.07												
10	22694							2.88	3.18										
11	22695							4.51	3.34										
12	22696							4.00	3.20										
13	22697									2.91	1.97								
14	22698									2.91	2.26								
15	22699									3.26	2.62								
16	22700											2.66	1.38						
17	22701											3.02	1.29						
18	22702											2.77	1.20						

19	22703													2.42	1.16				
20	22704													2.28	1.05				
21	22705													3.40	1.43				
22	22718															1.95	0.45		
23	22719															2.46	0.62		
24	22720															1.67	0.32		
25	22721																	1.10	0
26	22722																	1.57	0
27	22723																	1.50	0
Average		4.37	4.02	3.63	3.71	3.01	2.70	3.80	3.24	3.03	2.28	2.82	1.29	2.70	1.21	2.03	0.46	1.39	0
Relative increase in old serum		0.92		1.02		0.90		0.85		0.75		0.46		0.45		0.23		0	
Relative increase in young serum																			

serum. Similar experiments were made with cat serum and their results recorded in Tables III and IV, and in Text-figs. 3 and 4. The action of cat serum was not apparent until a concentration of 20 to 25 per cent was reached. Afterward, its action became stronger and no growth was observed when the concentration had reached 60 per cent.



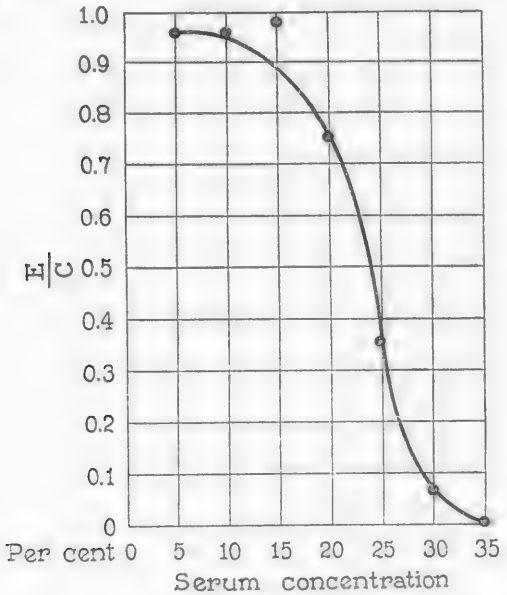
TEXT-FIG. 1. Experiment 1. Relation between the rate of growth of fibroblasts in dog and chicken sera. The ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

III.

Action of Heterogenic Sera from Young and Old Animals.

An examination was then made as to whether heterogenic sera from young and old animals would differ in their action on the growth of fibroblasts. Sera were obtained from cats 6 months and 9 years of age, and from dogs 6 months and about 10 years old. The tech-

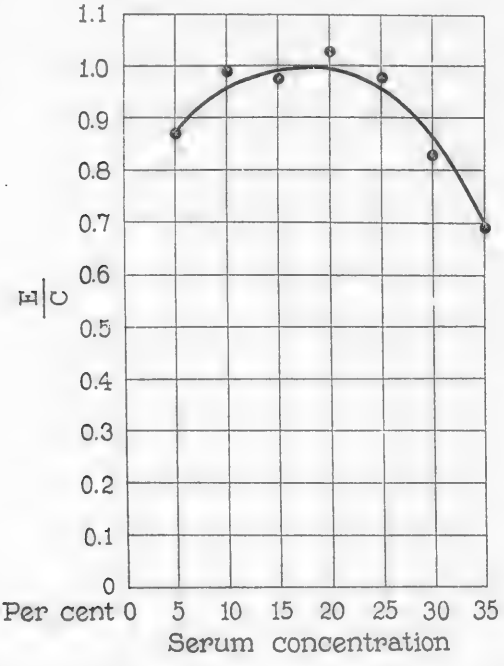
nique was identical with that previously described. The relative increases of the cultures of fibroblasts in young and old cat sera were compared. In Table V are recorded the relative increase of the tissue in both media, and the quotient of the figures expressing the rate of multiplication in the old and young cat sera. In Text-fig. 5, the ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, was plotted in ordinates and



TEXT-FIG. 2. Experiment 2. Relation between the rate of growth of fibroblasts in dog and chicken sera. The ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the serum concentration in abscissæ. The graph shows that the action of both sera was about the same as long as the concentration present in the medium was not above 20 per cent. Afterward, the growth decreased more rapidly in the old animal serum. When the concentration reached 50 per cent, the rate of growth was only 32 per cent of that observed in a medium containing a similar amount of young cat serum.

The same experiments were repeated with sera obtained from a dog 6 months old, and from another about 10 years old. The relative increase of the cultures of fibroblasts in both sera is recorded in Table VI and Text-fig. 6, in which the ordinates represented the quotient of the rates of growth in old and young sera, and the abscissæ



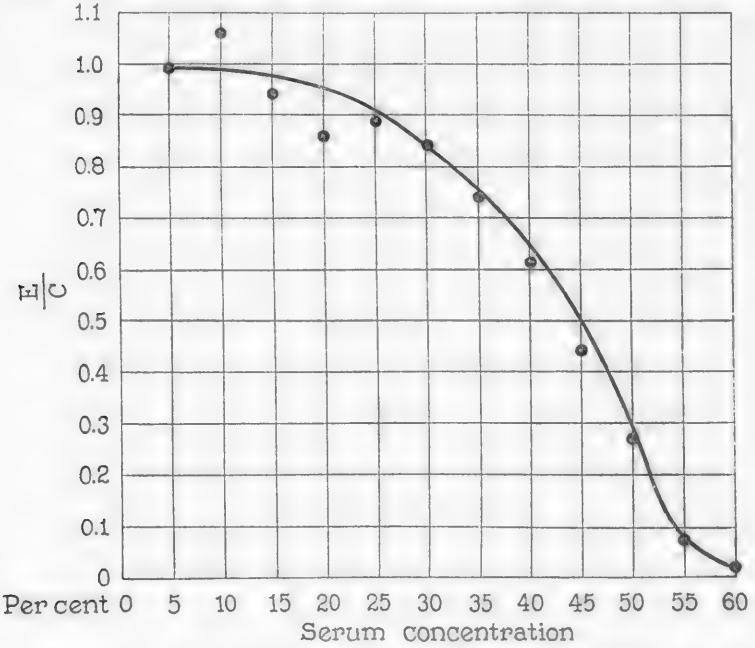
TEXT-FIG. 3. Experiment 1. Relation between the rate of growth of fibroblasts in cat and chicken sera. The ratio, $\frac{\text{Relative increase in cat serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the serum concentration in the medium. The graph shows that the inhibiting action of the old dog serum was more marked than that of the young dog serum. When the concentration of the serum reached 40 per cent, the rate of growth in the serum of the old animal was only 23 per cent of the rate of growth in the serum of the young one.

IV.

SUMMARY AND CONCLUSIONS.

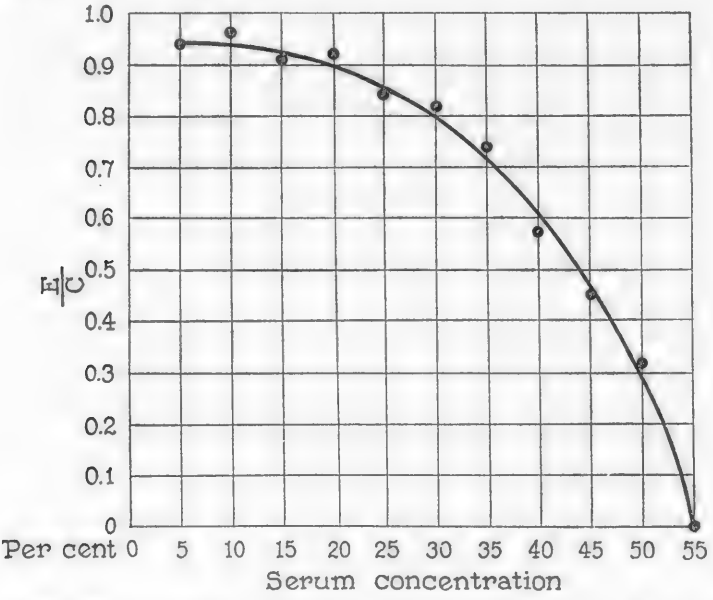
The presence in a culture medium of heterogenic serum of various concentrations exerts a definite influence on the rate of multiplication of fibroblasts. Dog serum does not inhibit the growth of



TEXT-FIG. 4. Experiment 2. Relation between the rate of growth of fibroblasts in cat and chicken sera. The ratio, $\frac{\text{Relative increase in cat serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

chicken fibroblasts markedly until its concentration reaches 15 per cent. Beyond this figure, each increase of the concentration brings about a rapid decrease in the rate of cell multiplication. When the concentration reaches from 30 to 45 per cent, no growth takes place. The inhibiting action of cat serum begins to manifest itself at a concentration of 25 per cent and prevents cell prolifera-

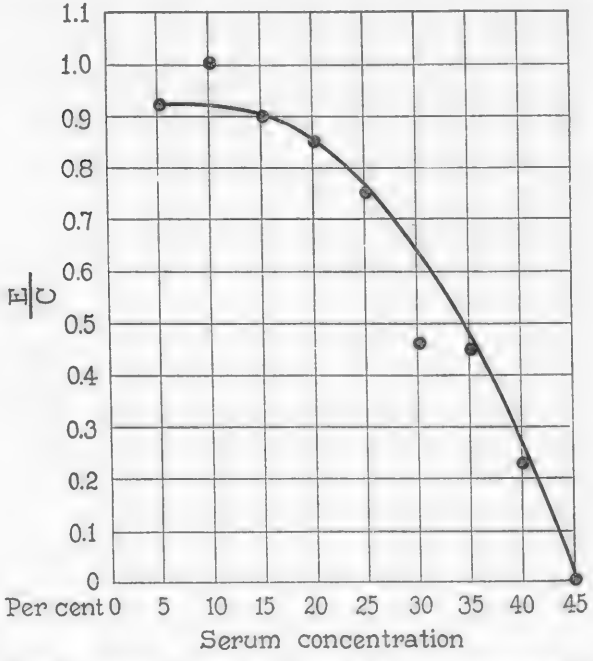
tion completely at a concentration of 55 and 60 per cent. The ratio, $\frac{\text{Rate of growth in heterogenic serum}}{\text{Rate of growth in chicken serum}}$, can be taken as expressing the action of the serum on fibroblast multiplication; that is, as the growth index of the serum.



TEXT-FIG. 5. Relation between the rate of growth of fibroblasts in young and old cat sera. The ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

The inhibiting influence of heterogenic serum was found to vary in direct ratio to the age of the animal from which it was obtained. The rate of proliferation of chicken fibroblasts was studied comparatively in media containing varied concentrations of serum from young and old animals. For each concentration of serum, the rate of growth in the serum of the old animal was expressed in relation to the rate of growth in the serum of the young animal. When cat

serum was used, the curve obtained in plotting this ratio in ordinates and the serum concentration in abscissæ showed a rapid increase in the inhibiting action of the old serum as soon as the concentration reached 30 per cent. The same tests were repeated with the serum from young and old dogs. The general results were identical, although



TEXT-FIG. 6. Relation between the rate of growth of fibroblasts, in young and old dog sera. The ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the quantitative inhibiting action of both sera was greater than that of cat serum. It may be concluded that under the conditions of the experiments:

1. Heterogenic sera inhibit and prevent the growth of chicken fibroblasts when their concentration is made to vary within certain limits.

2. A relation exists between the rate of growth of chicken fibroblasts, the concentration of the heterogenic serum, and the age of the animal from which the serum is taken. Thus, the rate of proliferation of chicken fibroblasts expressed by the growth index of the serum can be used as a means for detecting certain changes brought about by age in a heterogenic serum.

A STUDY OF THE RELATION OF TREPONEMA PALLIDUM TO LYMPHOID TISSUES IN EXPERIMENTAL SYPHILIS.

By LOUISE PEARCE, M.D., and WADE H. BROWN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 27, 1921.)

The establishment of the fact that dissemination of *Treponema pallidum* in the rabbit occurs after local inoculation has for the most part rested upon the evidence of the development of syphilitic lesions remote from the site of the primary lesion and upon occasional demonstrations of spirochetes in certain tissues, such as the spleen, blood, bone marrow, and lymph nodes. Our experience of the frequency of generalized lesions following inoculation of testicle or scrotum has differed from that of most workers on experimental syphilis, and in addition we have shown that it is possible to produce generalized lesions with great regularity by simple technical procedures either at the time of infection or shortly thereafter so that under certain conditions at least, dissemination of *Treponema pallidum* is known to have occurred in every animal infected (1). The mechanism of the process, however, is still undetermined, and before the question of a generalized infection can be put upon a logical basis, it is necessary that such essential facts as the time, frequency, and extent of dissemination under the usual conditions of inoculation procedure together with the localization and extent of the infection should be obtained. Among the possible modes or paths of dissemination which might be assumed to participate in the process of generalization, are the lymphatic and blood systems, and both are easily accessible to experimental investigation, as indicated in a preliminary communication (2).

Among the generalized manifestations of experimental syphilis in the rabbit, enlargement and induration of various lymph nodes are commonly observed. The superficial nodes which most frequently show various degrees of involvement after the usual scrotal or sheath inoculation are the inguinals, and similar changes are seen in the sacral retroperitoneal glands following testicular inoculations. In-

volvement of other nodes such as the flank, axillary, auricular, cervical, and submental glands has also been observed in certain animals, and apparently some abnormality of the popliteals is a constant feature of the infection irrespective of the site of inoculation.

Such changes as take place, however, might logically be expected to appear first in the lymph nodes draining the area of the primary reaction if dissemination of spirochetes from a local focus of inoculation regularly occurs by way of the lymphatics, and consequently the problem was first approached by a series of experiments designed to show the presence or absence of *Treponema pallidum* in the inguinal lymph nodes of rabbits inoculated in the scrotum.

The studies of the part played by the blood stream in the process of dissemination of *Treponema pallidum* are reserved for a later publication, but it should be stated here that before it could be concluded that organisms were localized in lymphatic tissue, it was necessary to exclude the blood as a possible source of spirochetes. During the early stages of the infection, this could be done only upon the basis of relative infectivity. Later, however, blood inoculations were uniformly negative. It is obvious, moreover, that a blood stream infection must be ruled out before one can conclude that spirochetes are localized in any other tissue, as for instance in the spleen or bone marrow.

EXPERIMENTAL.

The inguinal lymph nodes of normal rabbits are small, flattened or ovoid masses which are barely visible or palpable and are frequently imbedded in relatively large pads of fat which renders them more difficult of detection. Gross changes in the nodes are usually detected within 4 or 5 days after scrotal inoculation and consist in a slight enlargement together with an increased resistance or tension.[1] As the process continues, the glands rapidly become indurated and many times their normal size, and, in our experience, show some degree of abnormality as long as an active syphilitic process exists in the scrotum.

[1] This was the condition at the time these experiments were carried out. Subsequently, however, definite alterations of the nodes have frequently occurred within 24 hours after scrotal or sheath inoculation.

The demonstration of *Treponema pallidum* in lymph nodes may be accomplished by two methods—dark-field examination of fluid aspirated from the node or by animal inoculation. Direct aspiration of lymph nodes *in situ* by means of a syringe is a difficult procedure, particularly in the early involvement of the nodes, and the fluid obtained is often contaminated with blood. Moreover, as will be pointed out later, such a procedure may necessitate the examination of many preparations, and, therefore, we had recourse to the second and more satisfactory method which consisted in the removal of an inguinal node under ether anesthesia, the making of a node emulsion with salt solution, and the injection of this emulsion into the testicles of normal rabbits.

The extirpated lymph node is first minced with fine scissors and then ground in a sterile mortar with a small quantity of sterile normal salt solution. In most of our experiments we used a total quantity of 1.5 cc. of saline solution and were able to recover 1.2 cc. of the emulsion; 0.6 cc. was then injected into one testicle of each of two normal rabbits by means of a Fournier tuberculin syringe and a No. 22 gauge Luer needle. The subinoculated rabbits were closely followed during an observation period of 2 months for the development of syphilitic lesions. The organisms used in these experiments were the Zinsser and the Nichols strains of *Treponema pallidum* which have been maintained in rabbits for periods of 7 to 8 years.

Results.

The Frequency and Time of Inguinal Lymph Node Involvement.

Our preliminary experiments were concerned with the demonstration of the presence of *Treponema pallidum* in the inguinal lymph nodes of a few rabbits with large actively growing chancres. The results of the individual experiments are grouped together, and, as may be seen in Table I, the interval of time after scrotal inoculation at which the six experiments were carried out was progressively shortened from 61 to 16 days. The chancres were all well developed, while the inguinal glands were many times normal size, and markedly indurated. One gland was excised from each rabbit. Although spirochetes were found by dark-field examination in but one gland emulsion

a syphilitic orchitis developed in all the subinoculated rabbits in from 15 to 41 days as a result of the testicular injection of the lymphatic gland emulsion, showing that there had been an actual dis-

TABLE I.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 61 to 16 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	Interval between scrotal inoculation and excision of inguinal lymph node.	Examination of inguinal node emulsion for spirochetes.	Rabbit inoculated with lymph node emulsion.		Result of inoculation.	Incubation period of orchitis.
			No.	Testicle.		
	days					*days*
A	61	Negative.	1	Right.	+	41
				Left.	+	
B	49	"	2	Right.	+	Exact time
				Left.	+	not known.
C	35	Positive.	3	Right.	+	15
				Left.	+	33
D	35	Negative.	4	Right.	+	33
				Left.	+	33
E	18	"	5	Right.	+	27
				Left.	+	28
			6	Right.	+	26
				Left.	+	28
F	16	"	7	Right.	+	34
				Left.	+	34
			8	Right.	+	26
				Left.	+	28
Total..6						

+ indicates infection.

In the preliminary experiments, one rabbit was used for each gland emulsion and both testicles were inoculated, but later, the precaution of using two rabbits was adopted. In the majority of experiments, one testicle only was inoculated.

semination of spirochetes in each instance from the local focus of infection in the scrotum.

Our next experiment was planned to determine the incidence of infection of the inguinal nodes 7 days after scrotal inoculation, when

enlargement and increased resistance of the nodes are found in practically every instance, although the specific reaction about the implant is usually just beginning. The results of the experiment are shown in Table II.

TABLE II.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 7 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	Rabbit inoculated with lymph node emulsion.		Result of inoculation.	Incubation period of orchitis.
	No.	Testicle.		
				days
G	9	Right.	+	28
		Left.	+	28
	10	Right.	+	28
		Left.	+	28
H	11*	Right.	+	35
	12*	"	+	33
I	13*	"	+	35
	14*	"	+	35
J	15	"	+	30
	16	Accidental death.		
K	Spirochetes +; no inoculation.			
L	" +; " "			
Total...6				Average..32

* Animals of poor quality with very small testicles.

\+ indicates infection.

A series of six rabbits was operated upon and one of the inguinal glands removed. Dark-field examination of the gland emulsions showed actively motile spirochetes in two instances and no subinoculations were made from these; the remaining four emulsions were injected into the testicles of eight normal rabbits. The injection of one emulsion into both testicles of two rabbits resulted in an acute orchitis which developed simultaneously in 28 days in the four testicles, while the remaining three emulsions were injected into one testicle each of six rabbits. There was one accidental death soon after inoculation, but in the surviving five animals, a syphilitic orchitis developed in from 30 to 35 days and progressed actively in the usual fashion.

The consistently positive results of this experiment were most striking in that all six animals examined 7 days after scrotal inoculation were found to have infected inguinal glands in spite of the fact that a specific reaction about the scrotal implant could scarcely be detected.

In the next experiment, the interval of time between scrotal inoculation and extirpation of the inguinal node was shortened to 5 days (Table III).

The saline emulsions of ten inguinal glands were injected into the testicles of eighteen normal rabbits; in two instances, one subinoculation was made, but in eight, two rabbits were used. One bilateral inoculation was made. Spirochetes were not found by dark-field examination in any of the gland emulsions. A syphilitic orchitis, however, developed in seventeen of the eighteen test rabbits with an average incubation period of 30 days; in two instances, the orchitis was detected 25 days and in five instances, 28 days after inoculation. In sixteen of the eighteen rabbits, the reaction was of a relatively rapid progressive type, while in one rabbit with very small atrophic testicles, it was not particularly active and tended toward a localized area of induration. The orchitis which resulted from the single bilateral inoculation in the experiment developed in 33 days in one testicle and 39 days in the other, which was the longest incubation period in the series. In one animal a progressive orchitis failed to develop during the observation period of 8 weeks, and it is possible that this was an instance of an atrophic orchitis; the companion rabbit, however, inoculated with the same material, showed the usual reaction with an incubation period of 34 days.

The results of this experiment coincide to a remarkable degree with those obtained in the previous series and demonstrate in a considerable number of animals that the inguinal lymph nodes of rabbits inoculated in the scrotum only 5 days previously and at a time when no specific reaction about the implant was observed were constantly infective. In addition, although no spirochetes were demonstrated in the lymph gland emulsion by dark-field examination, the material was sufficiently potent to produce an actively developing syphilitic orchitis with an average incubation period of 30 days.

Finally, a similar experiment was undertaken 2 days after scrotal implantation when no gross alteration of the inguinal nodes was detected by palpation or by inspection, since the previous experiments had shown that enlargement and tenseness of the nodes, 5 and 7 days after scrotal inoculation, were constantly associated with the presence of *Treponema pallidum*.

TABLE III.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 5 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	No. of rabbit inoculated with lymph node emulsion.	Result of inoculation.*	Incubation period of orchitis.
			days
M	17	+	33
	18	+	30
N	19	+	28
O	20†	?	?
	21	+	34
P	22	+	33
Q	23‡	+	33 and 39
	24	+	28
R	25	+	25
	26	+	33
S	27	+	33
	28	+	25
T	29	+	33
	30	+	29
U	31	+	28
	32	+	28
V	33	+	30
	34	+	28
Total.....10	18		Average.....30

* Right testicle inoculated.

† Rabbit 20 was an old animal with small testicles, and the reaction was of the atrophic type.

‡ Rabbit 23 was inoculated into both testicles with positive results in 33 and 39 days.

+ indicates infection.

One inguinal node was excised from seven rabbits, five of which had been inoculated 2 days previously with the usual tissue implant and two with a testicular emulsion injected into the scrotum. Salt solution emulsions of the nodes were made as in the previous experiments and injected into the right testicle of twelve normal rabbits as shown in Table IV. Although no spirochetes were found in

TABLE IV.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 2 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	No. of rabbit inoculated with lymph node emulsion.	Result of inoculation.*	Incubation period of orchitis.
			days
W	35	+	28
	36	+	30
X	37	+	28
Y	38†	+	38
	39†	+	36
Z	40	+	29
	41	+	29
AA	42	+	29
	43	+	29
BB‡	44	+	30
	45	+	33
CC‡	46	+	33
Total.....7	12		Average.....31

* Right testicle inoculated.

† Rabbits 38 and 39 were old animals with atrophic testicles which probably accounts in part at least for the longer incubation time of the orchitis.

‡ Rabbits BB and CC were inoculated by a scrotal injection of an orchitis emulsion instead of the usual tissue implant.

+ indicates infection.

any of the emulsions by dark-field examination, a marked syphilitic orchitis developed in all subinoculated rabbits with an average incubation time of 31 days. The inguinal glands which were removed from rabbits with tissue implants produced an orchitis which was detected 28 and 29 days after inoculation, with the

exception of two old animals with atrophic and less reactive testicles in which the orchitis developed more slowly with incubation periods of 36 and 38 days. The orchitis produced by the injection of the lymph nodes obtained from the two rabbits inoculated with a tissue emulsion was slightly longer, developing in 30 and 33 days, but obviously a much larger series would be necessary before any final comparisons could be made between the relative infectivity of the nodes resulting from the two types of scrotal inoculation.

The outstanding features of this experiment are first, the demonstration that only 48 hours after inoculation of the scrotum, the inguinal nodes contain active *Treponema pallidum*, and second, that the treponemata were constantly present in the inguinal nodes at this time. It is also worthy of comment that although no spirochetes could be demonstrated in the inguinal gland emulsions by dark-field examination, the orchitis resulting from the injection of the emulsions was of an actively developing type with an average incubation period of 31 days, and in half the cases of 28 and 29 days.

Time of Establishment of a Generalized Infection.

The previous experiments have shown that active treponemata capable of producing a rapidly progressive orchitis were constantly present in the inguinal lymphatic nodes of twenty-nine rabbits, 2 to 61 days after scrotal inoculation.

The question that next arose concerned the time of establishment of a generalized infection, since the inguinal glands are so rapidly infected after scrotal implantation although the development of the specific chancre reaction itself is usually not recognizable for some 10 to 14 days (3). In reality it is conceivable that many parts of the animal body might be infected shortly after local inoculation and that they in turn might serve as other foci for further dissemination of the virus. At any rate, we know that the inguinal nodes contain active treponemata as early as 2 days after scrotal inoculation, but on the other hand, such organisms might be chance invaders which had been rapidly disseminated from the primary focus of infection and, as such, would be incapable of independently sustaining an infection. Since there was no available information on these points, the following experiment was undertaken.

A series of ten rabbits was inoculated with tissue implants in the right scrotum only. 2 days later, under ether anesthesia, the entire right testicle with the scrotum was excised, leaving only a narrow circular strip of the posterior scrotal skin with which to close the wound. Healing took place promptly in each animal with no secondary infection. At the time of operation, no difference in the two inguinal lymph nodes could be made out by palpation and no specific reaction about the implant had occurred. The rabbits were kept under careful observation and the results of the experiments are shown in Table V. 40 days after operation, and 42 days after scrotal inoculation, a definite syphilitic lesion was

TABLE V.

Results Following Unilateral Amputation of the Right Scrotum and Castration of the Right Testicle, 2 Days after Inoculation of the Right Scrotum.

No. of rabbit.	Development of syphilitic lesions in left testicle.	Interval between inoculation and appearance of testicular lesions.	Presence of popliteal lymphadenitis.	Lesions of generalized syphilis, other than testicular.	Interval between inoculation and appearance of generalized lesions.
		days			*days*
A	+	42	+	Bone.	49
B	+	42	+	Skin.*	42
C	+	49†	+	Bone.	49
D	+	49†	+	"	45
E	+	49‡	+	" § skin.	45
F	+	42	+	"	49
G	+	42	+	"	75
H	+	49†	+	"	56
I	+	49‡	+	"	45
J	+	49‡	+	"	45

* Scrotal lesion.

† Suspicious orchitis—42 days.

‡ Orchitis of 3 or 4 days standing on the 49th day.

§ Lesion, right tarsus.

present in the left testicle or head of the epididymis in four rabbits and a suspicious lesion in three others. By the end of the 7th week after inoculation, the left testicle of all ten rabbits was the seat of a progressive nodular orchitis. In addition, definite periosteal nodules of the nasal bones in four cases and a skin granuloma on the outer side of the right tarsus in one case were also found at the time; *i.e.*, 45 days after inoculation. Bone lesions were found in four other rabbits 4 days later and in another animal by the end of the 8th week, while the last rabbit in the group showed definite bone lesions 75 days after inoculation. Other generalized manifestations consisting of cutaneous granulomata were observed in five rabbits during the 2 months succeeding the appearance of the first lesions.

The right inguinal lymph nodes were definitely enlarged and resistant for some 10 days after the operation and then became shot-like in character. Both popliteal nodes were likewise persistently enlarged and indurated in each rabbit of the series, this change being pronounced by the time of occurrence of the metastatic testicular lesions.

The consistent results of this experiment demonstrate that 2 days after scrotal inoculation an active syphilitic infection is sufficiently established in the rabbit so that it is no longer dependent upon the focus of primary inoculation as a source of spirochetes.

Localization of Spirochetes in Lymph Nodes Other than the Inguinals.

Other superficial lymph structures such as the popliteal, cervical, submental, auricular, axillary, and flank nodes may also become enlarged and indurated during the course of syphilitic infection of the rabbit following local inoculation, but with the exception of regional lymph nodes in the drainage area of an active lesion, the popliteals show the most conspicuous and persistent abnormalities, of which a resistance or induration is usually the most marked feature. In many instances, the popliteals may be the only nodes in which gross abnormalities are detected. In order to demonstrate the presence of *Treponema pallidum* in lymph nodes other than the inguinals which showed these clinical changes, a number of animal inoculation experiments were carried out, of which the following four examples are typical.

Rabbit A.—Inoculated in the right testicle with an inguinal lymph node emulsion. A syphilitic orchitis developed and spirochetes were demonstrated in fluid aspirated from the testicle 41 days after inoculation. Both popliteals were enlarged and resistant, and 51 days after inoculation, the left node was resected under ether anesthesia, emulsified with salt solution, and injected into the right testicle of two normal rabbits. At this time, there were no generalized syphilitic lesions and no spirochetes were seen in the lymph gland emulsion by dark-field examination. One of the subinoculated rabbits died from accidental causes, but in the surviving animal, a syphilitic orchitis, in which spirochetes were easily demonstrated, developed, with an incubation period of 43 days.

Rabbit B.—Bilateral scrotal inoculation. At the time of operation, 14½ months later, there were active skin granulomata on the outer side of the right hind foot, left ankle, and nose, and extensive residual ocular changes, not, however, active at this time. Both popliteals were extremely large and rather resistant with

some fluctuation. The right node was aspirated with a syringe and 2 cc. of opalescent fluid flowed into the syringe without suction. No motile spirochetes were seen by dark-field examination of this fluid, of which 0.5 cc. was injected into the testicles of two normal rabbits. A syphilitic orchitis subsequently developed in both rabbits with incubation periods of 39 and 42 days.

Rabbit C.—Bilateral testicular inoculation, with the popliteal lymph node of Rabbit B. At the time of operation, 3 months later, there was a nodular orchitis of 46 days duration and a keratitis in the right eye. Right popliteal lymph node slightly enlarged and indurated. Left popliteal lymph node, however, which had been enlarged and indurated for some weeks, was resected under ether anesthesia, emulsified with salt solution, and 0.5 cc. was injected into the testicles of two normal rabbits. No spirochetes were seen in the emulsion by dark-field examination. An orchitis, however, was detected in the first subinoculated rabbit in 22 days, with subsequent demonstration of spirochetes, and progressed in an active manner. The second subinoculated animal was discarded because of a subcutaneous abscess.

Rabbit D.—Bilateral scrotal inoculation. At the time of operation, 16 months later, there were cutaneous, subcutaneous, and periosteal nodules of the lower portion of the nose and a large cutaneous granuloma of the left ankle. A right axillary lymph node and one in the upper left flank had been enlarged and indurated for some months. Enlarged cervical lymph nodes consisting of several firm, fairly elastic nodules in the deeper tissues of the neck and one large node under the right maxilla near the midline had also been observed for a considerable period of time. The large submental node was aspirated with a syringe and 0.4 cc. of an opalescent fluid was withdrawn. No spirochetes could be demonstrated by dark-field examination. The fluid was injected into the right testicle of a normal rabbit, and a nodular syphilitic orchitis subsequently developed with an incubation period of 51 days.

These experiments demonstrate that during the course of the syphilitic infection of rabbits produced by local inoculation of testicle or scrotum, superficial lymph nodes are infected with *Treponema pallidum*. In Rabbit A, there were no generalized lesions, and in Rabbit C, although there was a keratitis, there were no lesions about the hind feet and legs at the time the popliteal node was excised, so that the presence of *Treponema pallidum* in these nodes cannot be ascribed to a drainage infection. In the case of Rabbit B, however, there were active syphilitic lesions of the lower extremities and about the nose, so that the factor of drainage must be included in the infection of the regional lymph nodes in this instance. Clinically, this added factor is usually expressed by the larger size and the tenseness of the

lymph nodes rather than by induration. In Rabbit B, an axillary and an upper flank node were also enlarged and indurated, a condition which was undoubtedly due to the presence of *Treponema pallidum.*

Duration of Lymph Node Infection.

The presence or absence of spirochetes in lymph glands during the various phases of syphilitic infection in the rabbit is of particular interest and importance and especially during the so called latent period in which no syphilitic lesions can be found. As we have pointed out in a previous paper (4), spontaneous recovery in the rabbit is frequently only temporary, and lesions of some kind may recur after the lapse of a few months and occasionally of more than a year. Such intervals are examples of true latency in which the rabbit is still infected, as evidenced by subsequent syphilitic lesions, but during which the animal organism is able, for the time being, both to restrain the progressive development of spirochetes and to protect itself from their harmful effects. During this latent period, there is but one condition to indicate that infection may still exist, and that is the presence of some degree of abnormality of the superficial lymph nodes, especially the popliteals. The changes noted in cases of latent infection vary from a shotty induration of small nodes to a moderate enlargement with some degree of induration. The method of animal inoculation was used to ascertain whether spirochetes were present in the lymph nodes of rabbits in which the infection was latent.

The eight rabbits used for this series of experiments had been locally inoculated 7 months to $4\frac{1}{4}$ years previously as is shown in Table VI. All had had characteristic local lesions, and the first six animals in the table had shown well marked generalized lesions. The eighth animal was a female whose inoculation on the vulva resulted in a typical chancre, but no succeeding generalized manifestations had developed. At the time of operation, six rabbits showed no lesions of any kind, while two showed slight lesions of an indifferent character in which no spirochetes could be demonstrated by dark-field examination, and it was subsequently shown that one of these was not syphilitic (5). The latent periods of infection in these animals varied from 3 to 25 months. All eight rabbits, however, showed a popliteal adenopathy varying from small shot-like glands which were atrophic and

fibrous to moderately enlarged and indurated glands which showed some degree of medullary swelling. The popliteal nodes were resected under ether anesthesia, emulsified with salt solution, and 0.5 cc. of the emulsion was injected into the right testicle of two normal animals.

The results of these experiments demonstrate the constant presence of *Treponema pallidum* in the popliteal nodes of rabbits during latent periods of a syphilitic infection. Moreover, the rapidity with which an orchitis developed in the subinoculated rabbits, with incubation periods of 31 to 44 days, is particularly striking. In both of these respects, the results practically coincide with those obtained from the inoculation of lymph gland emulsions from cases of acute or active syphilis, as shown in the preceding sections of this paper. The duration of infection or of the period of latency in the group of animals reported is not unusual, except in Rabbits A, G, and H. The infection in these animals had at all times been mild; only a few lesions developed and these were comparatively slight. Nevertheless, virulent organisms remained alive in Rabbits A and G for approximately $4\frac{1}{4}$ and 3 years. Rabbit A is by far the longest case of animal infection on record and indicates very conclusively that the apparent recovery which takes place in these animals is only a symptomatic one.

The subsequent history of the eight rabbits extends over many months. Five animals have shown no change whatever in their condition. In Rabbit D, a slight infiltration about the right nostril extended to the left and eventually presented the typical appearance of syphilitic lesions in this locality, while in Rabbit E, a keratitis developed later. In the case of Rabbit A, the lesion in the scrotum proved to be a malignant growth from which extensive metastases occurred to the right and left inguinal and left flank lymph nodes, lungs, liver, spleen, kidneys, and bone marrow, leading eventually to marked emaciation, asthenia, loss of sphincter control, and the development of trophic ulcers about the anus, so that the animal was killed.

It would seem, therefore, from the striking results of these experiments, that for long periods of time, *Treponema pallidum* may be present in the lymph nodes of rabbits which have recovered from clinical manifestations of the disease even though no further manifestations of the infection occur, and further that the reservoir of the organisms during latent periods of syphilitic infection is lymphoid tissue.

The Use of Lymph Node Reservoirs as a Means of Demonstrating Infection.

The data obtained from the various series of experiments described in this paper indicate the fundamental rôle played by lymphatic tissue in syphilitic infection of the rabbit. We know that dissemination of spirochetes from a local focus of inoculation in the scrotum to the inguinal lymph nodes occurs extremely rapidly and that during the course of an infection *Treponema pallidum* may be recovered from other superficial lymph nodes as well. This may be accomplished not only during active phases of the infection but in the so called latent period in which no manifestations of the disease are found except abnormal popliteal lymph nodes which contain treponemata of surprising infectivity.

Up to the present time, there has been no means at one's disposal to ascertain whether or not infection still persists in a rabbit if no local recurrence or generalized lesions develop, except to hold the animal for a prolonged period of time for such manifestations to appear. This has been the method employed in judging the results of the treatment of the experimental infection, but the use of lymph node inoculations offers an illuminating as well as a time-saving procedure for determining the ultimate or curative effect of a therapeutic agent, as illustrated by the following experiment.

Among a group of rabbits treated with arsphenamine and neoarsphenamine, there were five which showed no clinical recurrence during a period of 3 months observation. These animals had been inoculated in both testicles with the Nichols strain of *Treponema pallidum*; two rabbits were given single doses of 6 mg. per kilo of arsphenamine (German salvarsan Lot A 25819) and three rabbits 9 mg. per kilo of neoarsphenamine (German neosalvarsan, Lot A 25884). The drugs were administered intravenously in a 0.2 per cent solution; the arsphenamine was neutralized by the theoretical amount of N sodium hydroxide to form the disodium salt. The doses employed have been shown to produce definite therapeutic effects in the average animal, consisting of a regression of lesions approximating complete resolution with freedom from recurrence for 4 to 6 weeks followed by clinical relapse within a period of not more than 2 to 3 months (6).

The immediate therapeutic effects consisted in the regression and resolution of the testicular lesions, which proceeded somewhat more rapidly in those animals treated with neoarsphenamine, so that by

TABLE VI.

Demonstration of Treponema pallidum in the Popliteal Lymph Nodes during Latent Periods of Infection.

Source rabbit.	Duration of infection.	Length of latent period.	No. of rabbit inoculated with popliteal lymph node emulsion.	Result of inoculation.	Incubation period of orchitis.
	mos.	*mos.*			*days*
A*	51	6	1	+	38
			2	+	43
B	9	3	3	+	42
			4	+	44
C	7	3	5	+	37
			6	+	39
D*	9	3	7	+	34
			8	+	31
E	7	3	9	+	37
			10	Dead.	
F	7	3	11	+	31
			12	+	31
G	35	25	13	+	35
			14	+	35
H	10	7	15	+	34
			16	+	34

* These two animals showed suggestive lesions at the time of examination. They subsequently increased but no spirochetes could be demonstrated in them by dark-field examination. The lesion in the case of Rabbit A proved to be a malignant growth.

+ indicates infection.

the end of the 2nd week, the testicles were normal except for small residual areas of thickening or infiltration in four animals, which continued unchanged during the 3 months observation period following treatment.

Aspiration of the testicles for dark-field examination was not done because of the possible regressive effect of such a trauma. In addition no generalized manifestations of a specific character had developed. The only suggestive evidence of the existence of a still active infection 3 months after treatment was an enlargement and induration of the popliteal lymph nodes which were present in all five rabbits. In order to determine definitely the presence or absence of infection, the left

TABLE VII.

Demonstration of Treponema pallidum in the Popliteal Lymph Nodes 3 Months after Treatment with Arsphenamine and Neoarsphenamine.

Source rabbit.*	Interval between inoculation and treatment.	Treatment; amount per kilo.	Amount of popliteal lymph node emulsion injected.	No. of rabbit inoculated with popliteal lymph node emulsion.	Result of inoculation.	Incubation period of orchitis.
	days		*cc.*			*days*
A	18	6.0 mg. of arsphenamine.	0.3	1	+	37
			0.3	2	+	45
B	18	6.0 " " "	0.3	3	+	34
			0.3	4	+	34
C	18	9.0 mg. of neoarsphenamine.	0.4	5	+	34
			0.4	6	+	34
D	18	9.0 " " "	0.2	7	+	29
			0.2	8	+	31
E	18	9.0 " " "	0.4	9	+	29
			0.4	10	+	31

* All five rabbits presented a progressive nodular orchitis which was approximately at the height of its acute phase or cycle at the time of treatment.

+ indicates infection.

popliteal node was excised for inoculation into test animals. Upon removal, the nodes showed a slight degree of enlargement, slight fibrosis of the surrounding tissue, and slight thickening of the capsule; they were translucent and firm with prominent lobulation. A salt solution emulsion of each node was prepared and from 0.2 to 0.4 cc. was injected into the right testicle of two normal rabbits as shown in Table VII.

The noteworthy features of this series of experiments are first, the constancy with which positive results were obtained in each testicular subinoculation, showing that *Treponema pallidum* was present in the popliteal lymph nodes of each rabbit, and second, the infectivity of the treponemata as shown by the rapidity with which the orchitis developed. In eight instances, a testicular lesion was recognized in 29 to 34 days with an average incubation period of 32 days, while in two rabbits, the orchitis developed somewhat more slowly—37 and 45 days. These results are strikingly similar to those obtained from test inoculations of popliteal lymph nodes of latent cases of syphilitic orchitis and are in close approximation to those observed from the inoculation of inguinal lymph nodes of cases with active scrotal lesions.

It might be briefly stated that the three rabbits treated with neoarsphenamine and one of the two treated with arsphenamine subsequently showed an outspoken clinical relapse but that no characteristic lesions developed in the other during a 4 months period of observation following treatment.

The possibility of the recovery of spirochetes from lymph node reservoirs is further emphasized by the results obtained from the continued passage of *Treponema pallidum* from lymph node to testicle. For about 18 months we have carried the Nichols strain of *Treponema pallidum* in eight serial inoculations of inguinal or popliteal lymph nodes to testicles of normal rabbits, thus demonstrating the ability to recover spirochetes from lymphatic tissue through successive generations. With such a procedure, the orchitis tends toward a more prolonged or chronic type with a distinctly longer incubation period which averaged from 4 to 6 weeks. Not infrequently, the reaction consisted in a primary atrophy of the testicle rather than an enlarged granulomatous change. That such a condition is one type of primary response toward *Treponema pallidum* is shown by dark-field demonstration of spirochetes. Moreover, in doubtful cases when no lesions of the testicle can be detected by palpation, infection may be recognized by an adenopathy of the superficial lymph nodes.

Finally in this connection, reference should be made to the use of lymph node reservoirs for preserving stock strains of *Treponema pallidum* when not in active use, thus obviating the necessity of frequent testicular or cutaneous transfers (7).

DISCUSSION.

The studies reported in the present paper were undertaken for the purpose of ascertaining the relation of *Treponema pallidum* to lymphoid tissues in syphilis of the rabbit produced by local inoculation. The problem was approached by a series of experiments designed to show the frequency, time, and extent of lymphatic dissemination of spirochetes from a primary focus of infection in the scrotum or testicle, and the method employed consisted of the demonstration of *Treponema pallidum* in the lymph nodes of infected rabbits by animal inoculation.

The first series of experiments was carried out with emulsions of satellite nodes from rabbits which had been inoculated in the scrotum 2 to 61 days previously. Nodes from twenty-six animals produced a syphilitic orchitis when injected into the testicles of normal rabbits, and spirochetes were found in three others, thus demonstrating that a dissemination of organisms from a primary focus of inoculation constantly occurs by way of the lymphatic system. In only one out of forty-six rabbits inoculated did a progressive orchitis fail to develop, and in this instance, the orchitis was of the atrophic type. In contrast to the high infectivity of the node emulsions is the small number of spirochetes that must have been present in the nodes, since organisms were demonstrated by dark-field examination in only three out of twenty-nine emulsions.

The lymph node emulsions contained little or no microscopic blood, and, in addition, parallel experiments have shown that inoculations of as much as 0.5 cc. of blood rarely produce an infection with an incubation period of 5 weeks.

The second point of importance emphasized by the results of these experiments is in regard to the time of dissemination of the spirochetes from the local focus of inoculation in the scrotum. At the time this work was done, the inguinal lymph nodes showed definite changes, consisting of an enlargement and tenseness or resistance within 4 or 5 days after scrotal inoculation and ultimately progressed to a condition of marked enlargement and induration. With the knowledge that has been gained of the constant dissemination of spirochetes from the scrotum, at least as far as the satellite nodes are concerned, one might logically assume that the early changes in the nodes

signified the presence of spirochetes. That this assumption is justified, has been borne out by the consistently positive results of the series of rabbits in which the inguinal nodes were removed 5 and 7 days after scrotal inoculation. The striking feature in regard to the time of dissemination, however, is revealed by the constant infectivity of the inguinal lymph nodes only 2 days after scrotal inoculation, at a time when no gross abnormalities were detected, and, in addition, when no syphilitic reaction about the chancre implant could be recognized. Moreover, it seems justifiable to assume from the evidence at our disposal that enlargement and tenseness of the inguinal lymph node occurring at present as early as 24 hours after scrotal or sheath inoculation are due to the presence of *Treponema pallidum*.

The character or degree of the infection present in the nodes as indicated by the type of orchitis produced by the inoculation of the node emulsions is of considerable interest. There were 57 inoculations made, four of which are omitted from the present analysis (accidental death, etc.). The average incubation period of the orchitis which occurred in the remaining 53 animals determined by the development of palpable lesions was 31 days; in twenty-five animals, it was 29 days or less, and the longest incubation period was 41 days (two animals). Examination of testicular fluid would doubtless have shown the presence of an infection much earlier, but was not carried out on account of the traumatism which may obscure subsequent changes.

It has further been shown that within 48 hours after scrotal inoculation spirochetes have become disseminated and sufficiently established in the animal body to maintain an active progressive infection in the event of the removal of the scrotum with the implant and testicle. Syphilitic lesions of the remaining testicle and epididymis developed in each of the ten rabbits comprising the series in from 40 to 46 days after operation, and, in addition, well marked generalized lesions in each animal were a striking feature of the experiment. These results are not to be interpreted as implying that the inguinal lymph nodes constitute the only focus of infection under the conditions of the experiment, but since it has been shown that spirochetes are constantly present in the inguinal nodes 2 days after scrotal

inoculation, it may be assumed that they serve as one such focus. In any event, this experiment demonstrates that the infection itself is so established within 2 days after scrotal inoculation that it is independent of the primary lesion as a source of spirochetes.

The general tendency of *Treponema pallidum* to localize in lymphatic tissue is also shown by the recovery of organisms from other nodes, such as the popliteal and submental. It further appears that such localization is not dependent upon an active lesion in the drainage area of the node but that a widespread dissemination of spirochetes from the local focus of infection occurs by way of the lymphatics. Thus, enlargement and induration of various lymph glands which may occur during the course of a syphilitic infection are significant of the presence of *Treponema pallidum*, and since a popliteal adenitis is a constant feature of syphilis in the rabbit, this condition may be considered as pathognomonic of infection.

The persistence of active spirochetes in the animal body for long periods of time without manifestations other than some abnormality of the popliteal nodes is a fundamental concept of syphilitic infection in the rabbit. We have shown that in cases of true latency of from 3 to 25 months duration, spirochetes may be recovered from the popliteal nodes by animal inoculation. The noteworthy features of this series of experiments were the constancy with which the nodes were shown to harbor active treponemata and the rapidity with which the resulting orchitis developed, the incubation period varying from 31 to 44 days. These results, moreover, are practically the same as those obtained by the testicular inoculation of inguinal lymph nodes from early or active cases of infection.

It would appear, therefore, that although the rabbit possesses some type of mechanism during latent periods of the infection which operates to protect the animal from the harmful toxic effects of the organisms and prevents the development of manifestations of the disease, the organisms themselves are not destroyed but are existent in lymphatic tissue in an active state. Whether other tissues may serve as similar foci for the maintenance of active treponemata for long periods of time is not known, but the well known affinity exhibited by *Treponema pallidum* for lymphatic tissue, together with the clinical evidence of continued adenopathy in latent cases of infection, would

point toward this tissue and especially its larger aggregations—the lymphatic nodes—as the principal reservoirs of the virus.

A close similarity existed between the orchitis produced by lymph node inoculation and that caused by an emulsion of either an infected testicle or a skin nodule such as is frequently employed in propagating *Treponema pallidum*. The incubation period of the orchitis produced by inoculation of a node emulsion was about 7 to 10 days longer, but once the process had reached the stage when it could be diagnosed by palpation, its further development proceeded in much the same manner and at about the same speed. This is the more remarkable when the spirochete content of the two inoculating emulsions is considered, spirochetes being numerous in emulsions prepared from a testicular or skin nodule, while it is unusual to see a single organism in those from a lymph node. Thus we are forced to the conclusion by the results of these experiments that actual numbers of spirochetes may play a very small rôle as a causative factor in producing an active progressive syphilitic orchitis and that the state of vitality or degree of infectivity of the spirochetes is of much greater importance.

The ability to recover spirochetes from lymphatic tissue is further emphasized by the results of the serial passage of lymph node emulsion to testicle. During the last 18 months, there have been eight transfers of this type, and in every instance it has been possible to produce a syphilitic orchitis by the inoculation of a lymph node emulsion, the infection of which had in turn resulted from a testicular injection of a lymph node emulsion.

The facts derived from this group of experiments may be profitably utilized in many ways. We have suggested a simple method based upon the duration of lymph node infection for the preservation of stock strains of *Treponema pallidum*, which is both economical and time-saving, and, in addition, is especially effective in guarding against loss of the strain.

A further utilization of the facts derived from these experiments lies in the demonstration of infection in rabbits which show no manifestations of disease as a result of the administration of therapeutic agents. The methods commonly employed in judging the results of therapy have consisted in observations on the effect of the agent upon the spirochetes and upon the regression, healing, and recurrence of

the local lesion during observation periods of from 1 to 3 months. In our experience, however, we have seen many instances of a relapse in regions remote from the original lesion with no local recurrence, and, in addition, we know that either a local or a generalized relapse may occur considerably longer after treatment than 3 months. Such rabbits may show a characteristic popliteal adenitis, or there may be no distinctive adenitis, but with the knowledge which has been gained of the persistence of *Treponema pallidum* in lymph node structures, it is possible to demonstrate the presence of spirochetes by animal inoculation without waiting for a clinical relapse. This was done in the case of five rabbits treated with arsphenamine and neoarsphenamine which had shown no local or general manifestations of disease during the 3 months observation period following treatment. The popliteal nodes excised from these rabbits were inoculated into the testicles of normal rabbits with the production of a syphilitic orchitis in all instances. Subsequently four of the five rabbits showed typical local recurrences.

SUMMARY.

A widespread dissemination of *Treponema pallidum* from a local focus of inoculation in the rabbit constantly occurs by way of the lymphatics. Spirochetes were regularly recovered from the satellite lymph nodes by animal inoculation after scrotal inoculation; they were present as early as 2 days, when no specific primary reaction was detected, and at later periods of from 5 to 61 days after inoculation. Other superficial nodes at remote sites such as the popliteals and with no syphilitic lesions in the drainage area have also been shown to harbor active organisms. Although spirochetes were found in relatively few of the lymph node emulsions, the orchitis resulting from their injection was of a rapidly progressive type with an incubation period but slightly longer than that produced by a testicular or skin nodule emulsion rich in spirochetes.

It has further been shown that a syphilitic infection is sufficiently established in the rabbit body within 48 hours after scrotal inoculation so that the primary lesion is no longer essential for its maintenance.

Active treponemata survive in the popliteal lymph nodes for long periods of time and have been regularly recovered from them in cases

of true latency. The lymph nodes, therefore, function as reservoirs of the organisms. The ability to recover the spirochetes from lymphoid tissue through successive generations is seen in the serial passage of lymph node emulsion to testicle during an 18 months period.

The persistence of spirochetes in lymphoid tissue irrespective of the presence or absence of syphilitic lesions is a characteristic and fundamental feature of syphilis of the rabbit. The existence of infection, therefore, may be demonstrated at any time by the recovery of spirochetes from the popliteal lymph nodes by animal inoculation. This fact is of great practical importance in the therapy of the infection and may be profitably utilized in determining the ultimate effect of a therapeutic agent.

These experiments demonstrate that the disease is not confined to the site of local inoculation but that lymphogenous dissemination of treponemata regularly takes place, and that during the course of this process organisms become localized in the lymph nodes and exist there indefinitely irrespective of the occurrence of manifestations of disease. The intimate relation of *Treponema pallidum* to lymphoid tissue is an essential concept of syphilis of the rabbit, and from this point of view, the infection is primarily one of lymphoid tissue.

BIBLIOGRAPHY.

1. Brown, W. H., and Pearce, L., *Arch. Dermatol. and Syphilol.*, 1921, iii, 254.
2. Pearce, L., and Brown, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 164.
3. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 709.
4. Brown, W. H., and Pearce, L., *Am. J. Syph.*, 1921, v, 1.
5. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 201.
6. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1921, xxxiii, 553.
7. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1921, xxxiv, 185.

STUDIES ON AGGLUTINATION WITH THE AID OF THE CENTRIFUGE. THE INFLUENCE OF TEMPERATURE ON ABSORPTION AND FLOCCULATION.

By FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 13, 1921.)

Whatever opinions may be held regarding the nature of the specific agglutination of bacteria it is generally agreed that the process occurs in two phases; a reaction between cells and serum, in which the antigen and the specific antibodies combine, and a secondary reaction among the affected bacteria in which they cohere and flocculate out of suspension.[1]

Both phases of agglutination proceed gradually to completion and are subject to variation according to the physical conditions under which they occur. Thus, temperature, agitation, mass action, and the influence of electrolytes are known to affect the phenomenon. In the usual agglutination technique the spontaneous flocculation of the bacteria serves as the index of the primary reaction between cells and serum. The complete process, however, includes all the variable factors to which the flocculation phase is subject, and thus the conditions under which the absorption phase proceeds are not open to separate analysis. A method of examination which would eliminate the unknown time element in the flocculation phase, and thus afford a cross-section view of the status of the absorption phase at any period in its progress, might be of value in a closer analysis of some of the factors affecting absorption and perhaps throw some light on the nature of the reaction. The flocculation of bacteria in the presence of their specific antisera may be mechanically effected by means of the centrifuge. The affected organisms, brought into contact by centrifugation, tend to adhere in clumps identical with those produced by the unaided process of agglutination.

[1] For the purposes of this paper the current terms absorption and flocculation have been accepted for these phases of the reaction.

Gaehtgens[2] (1906) advocated the use of the centrifuge in agglutination tests with *B. typhosus*. Small, round bottomed tubes of serum and bacterial suspension were centrifuged for 10 minutes without previous incubation, and the resulting sediment was examined macroscopically and microscopically for evidence of clumping. In positive instances a characteristic sediment was formed, which broke up into the typical flocculi of agglutination on shaking, and on microscopic examination showed large clumps of adherent bacilli. The results of centrifuge agglutination with the sera of 100 persons duplicated the findings by the usual method. Control sera, producing no agglutination in the incubator, gave uniformly negative results with the centrifuge. In 1907 and 1908, Brian[3] and Gaehtgens[4] applied the centrifuge method to meningococcus agglutination, which requires 16 to 24 hours incubation at 55°C. by the standard method.

In spite of obvious advantages in point of time and of more exact control over the conditions of test, Gaehtgens' method seems to have attracted little attention. Its failure to find a place among bacteriologic methods may have been due in part to the technical disadvantage of centrifuging the large numbers of tubes often used in agglutination tests, and in part to the confusion which attended the serological diagnosis of the meningococcus—a field in which it might have been useful—before the variations in agglutinin response had been explained by Dopter. Gaehtgens' method seems to have survived for a time only in the diagnosis of glanders, and to have fallen into disuse.

During the period of the war we employed the centrifuge method of agglutination in an extensive series of diagnostic and analytical experiments with the meningococcus group of organisms. In many parallel tests the method was found to be entirely reliable, giving results closely in accord with those obtained by the standard method of incubation at 55°C. for 16 hours.

In the course of these experiments a number of observations were made bearing on certain physical conditions which affect the agglutination reaction. Most of these observations were made on the behavior of meningococci in their specific homologous antisera. These organisms proved to be well adapted for such studies because of the slow rate at which agglutination ordinarily proceeds. The experiments which dealt primarily with the effect of temperature on absorption and flocculation have been collected in this report.

[2] Gaehtgens, W., *Münch. med. Woch.*, 1906, liii, 1351.
[3] Brian, O., *Centr. Bakt., Ite Abt., Orig.*, 1907, xliii, 745.
[4] Gaehtgens, W., *Arch. Hyg.*, 1908, lxvi, 377.

The Centrifuge Method of Agglutination.

A standardized technique was maintained throughout except as noted in single experiments. The essentials of the method are as follows:

The meningococcus suspensions used were heat-killed cultures which were found to behave in a uniform manner over a period of months.[5] The organisms were grown on glucose agar in Blake bottles for 16 hours at 37°C., washed off with 10 to 20 cc. of sterile 0.8 per cent salt solution, sealed in small bottles, and immediately heated to 65°C. for 30 minutes in a water bath. These concentrated stock suspensions, containing 20 to 50 billion organisms per cc., were stored at 4°C. without a preservative. In some instances the straw-colored supernatant fluid was replaced with sterile saline solution. Dilutions of 2 to 4 billion cocci were made and standardized[6] as required.

Immune serum of rabbits or horses was also stored at 4°C. without a preservative and the successive dilutions were made up fresh for each experiment.

Usually 1 billion organisms were suspended in 1 cc. of dilute serum. The tests were carried out in round bottomed test-tubes, 9 by 90 mm., without lips. They were incubated in a water bath or thermostat, as required by the conditions of the experiment, and centrifuged in groups of five to seven in the wide cups used in the Babcock milk test. An eight cup head on the electric centrifuge thus accommodated 40 to 56 tubes at one time. The specimens were uniformly centrifuged at a speed just sufficient to deposit unagglutinated organisms—10 minutes at 1,800 revolutions per minute.

After centrifugation the tubes were agitated to bring the sediment into suspension and the results read macroscopically by oblique transmitted light against a dark background. Examined in this manner the evidences of agglutination are unmistakable. Unagglutinated organisms, in normal serum or in salt solution, on centrifugation form a compact button in the bowl of the tube. On shaking slightly the sediment comes up in a smooth corkscrew whirl and is evenly distributed throughout the liquid. In the presence of an active serum, however, the central button is surrounded by a fringe of feathery flocculi spread over the bottom of the tube. This flaky layer was well described by Gaehtgens.[2] On shaking, the sediment rises in small clumps or larger masses in a clear or turbid fluid, depending upon the degree of agglutination present. The appearance is identical with that found after spontaneous flocculation. In our experiments three degrees of agglutination were recognized: complete, all the organisms compactly clumped in a clear liquid; incomplete, abundant clumps in a slightly hazy liquid; and partial, small but distinct clumps in a cloudy liquid. In the

[5] Amoss, H. L., Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1920, xxxii, 778.
[6] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

charts these states are indicated by black bands of different widths, the broad bands indicating complete agglutination. The experiments were carried out with the usual controls in normal serum and salt solution.

Comparison of Agglutination of Meningococci by the Standard and the Centrifuge Methods.

When Brian and Gaehtgens published their observations nothing was known of the specific variations among meningococci which led to the establishment of types by Dopter and their confirmation by Gordon and by Nicolle, Debains, and Jouan. The first problem, therefore, in the application of the centrifuge method to immunological studies with meningococci was to examine the method for type specificity in comparative tests with the standard agglutination technique.

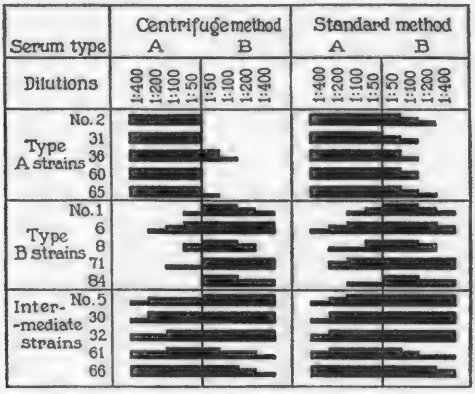
This has been done repeatedly in many experiments with meningococci of spinal or nasal origin, in normal sera and in monovalent or polyvalent antisera from the rabbit or the horse. Typical examples of such comparative tests are shown in Text-fig. 1, for the two main types of meningococci, Types A and B,[7] and for strains which on agglutination show an intermediate character, coming down in almost equal dilutions of the antisera of each type.

Experiment 1.—Type A serum, Horse 33. Type B serum, Horse 32. Sera 1 month old. Serum dilutions 1:50 to 1:400. 1 billion heat-killed organisms suspended in 1 cc. of dilute serum. Duplicate sets. One set centrifuged immediately, shaken, and read. The other set incubated 16 hours at 55°C., shaken, and read. Results in Text-fig. 1.

In this experiment it may be seen that centrifugation without preliminary incubation gives somewhat more clear-cut results in Groups A and B, while in the group of intermediate strains the results by both methods are practically identical. The specific immunological differences among meningococci are thus shown to be discernible by this method, and indeed are accentuated rather than decreased, so that the centrifuge method is, perhaps, the more useful

[7] Following Dopter we accept the classification of Nicolle, Debains, and Jouan, in which the meningococcus is designated Type A, and Dopter's parameningococci, alpha, beta, and gamma, are called Types B, C, and D respectively.

for type diagnosis. These differences are probably due in part to the fact that the so called coagglutinins seem to react more slowly than do the specific agglutinins and require a longer incubation time for their demonstration. A discussion of this interesting difference is not within the scope of this paper.



TEXT-FIG. 1. Centrifuge agglutination of meningococci without incubation *versus* the standard method, incubated 16 hours at 55°C.

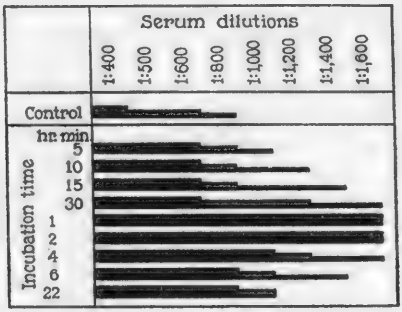
The Effect of Incubation at 55°C. on the Serum Titer.

In general, centrifuge agglutination without incubation has given results of somewhat lower titer than has spontaneous agglutination after 16 hours incubation at 55°C. This fact led us to combine a short incubation period with the mechanical flocculation, and in this way the highest titers with a given serum were obtained. These observations were based on experiments such as the following.

Experiment 2.—Type B meningococcus, Strain 1, agglutinated in homologous monovalent antiserum from a rabbit. Suspensions of the killed culture, in successive dilutions of the antiserum, were centrifuged without incubation to determine the approximate limits of agglutination. Beginning with serum dilutions near the upper limit of complete agglutination, coccus-antiserum mixtures were set up at close dilution intervals, incubated at 55°C. for periods of 5 minutes to 22 hours, centrifuged, shaken, and read. The results are given in Text-fig. 2.

A period of incubation promotes absorption to such an extent that complete clumping occurs in dilutions two to four times higher than

without previous incubation. Even 5 minutes exposure to 55°C. has an obvious effect. The highest serum titer is found after 1 to 4 hours incubation. Long exposure, 16 to 24 hours, to 55°C. may cause a diminution in the titer. This observation has been made repeatedly and is due to a deleterious effect of the high temperature on the serum antibodies, as will be shown presently. Repetitions of Experiment 2 with other strains and sera have given practically identical results.



TEXT-FIG. 2. The effect of incubation at 55°C. on the titer of agglutination in antimeningococcus serum.

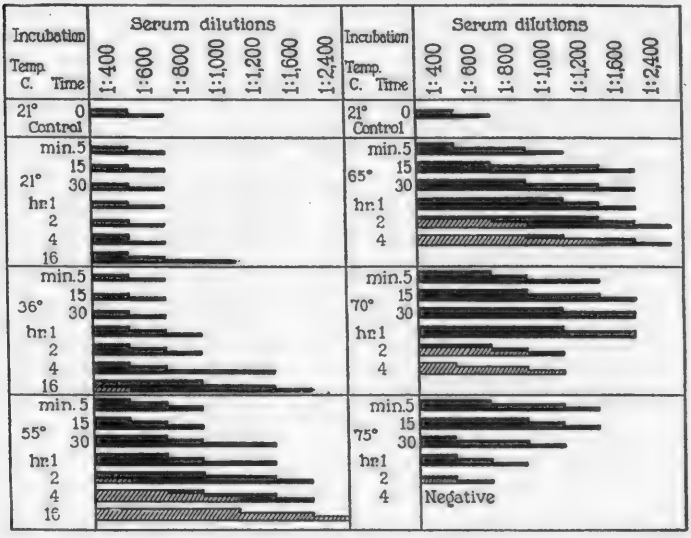
The Effect of Temperature on Absorption.

The foregoing experiment shows that contact of organisms and antisera for 1 to 4 hours promotes a reaction sufficient to produce clumping on centrifugation in high dilutions of the antiserum. Is the temperature at which the reaction takes place a function of the time required for its completion?

Experiment 3.—Suspensions of a Type B organism, Strain 1, were incubated at various temperatures and for varying periods of time in successive dilutions of a homologous monovalent antiserum from a rabbit. After the determined incubation period (water baths) the tubes were read to note the occurrence of spontaneous flocculation, and were then centrifuged, shaken, and read. The results are given in Text-fig. 3.

In the preliminary test, without incubation, complete agglutination occurred at 1:200 (not shown on the chart) with the limit of visible clumping at 1:600. In these serum dilutions the bacteria

absorbed enough agglutinin during their passage to the bottom of the tube to cause them to cohere in spite of resuspension. This reaction is a constant in each experiment and forms the base-line in each test. No more absorption than this occurred at 21°C. after 4 hours contact, but after 16 hours some agglutination was found in a dilution of 1:1,000. At 36° absorption proceeded more rapidly and a positive reaction was evident after a contact of 1 hour. As already noted

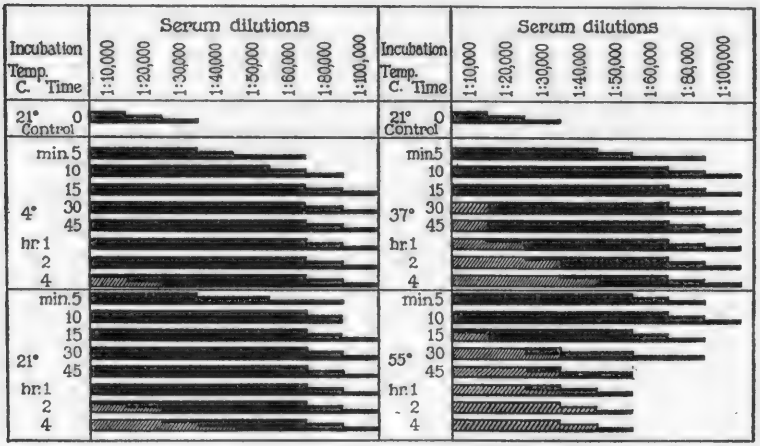


TEXT-FIG. 3. The influence of temperature on the absorption of meningococcus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

in Experiment 2, 5 minutes contact at 55° promotes absorption, which, however, did not reach the limit, 1:2,400, until more than 4 hours had elapsed. At the higher temperatures the effect of heat is more quickly apparent, so that at 65° the limit of the reaction was reached in 2 hours and at 70° and 75° the maximum absorption occurred at 30 and 5 minutes respectively. At these high temperatures the serum antibodies were already in process of destruction so that the titer fell off rapidly as incubation was prolonged. Similar

results were obtained in a parallel experiment with a Type A meningococcus in its antiserum.

Although the agglutination reaction is not well adapted to accurate quantitative estimation because the end-point is not sharp, it is interesting to note in these experiments that the time and temperature relations are roughly those of chemical acceleration. The behavior of the serum is also similar to that of enzymes, which are most active just below the temperature of rapid destruction.



TEXT-FIG. 4. The influence of temperature on the absorption of typhoid bacillus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

It is only with an organism such as the meningococcus which reacts with its antiserum at a measurable rate that such an experiment can readily be performed. *Bacillus typhosus* reacts with its specific antiserum so rapidly that the process seems to be complete in 15 minutes at 4°C. and in 10 minutes at 36°C.

Experiment 4.—Suspensions of heat-killed typhoid bacilli in high dilutions of antiserum from a horse. Identical sets of tubes incubated at various temperatures for varying periods of time. Read to note spontaneous flocculation, centrifuged, shaken, and read. The results are given in Text-fig. 4.

High temperature quickly showed an injurious effect on the *Bacillus typhosus* agglutinins. The titer of the serum began to fall off after an exposure of 10 minutes at 55°C.

The Effect of Temperature on Flocculation.

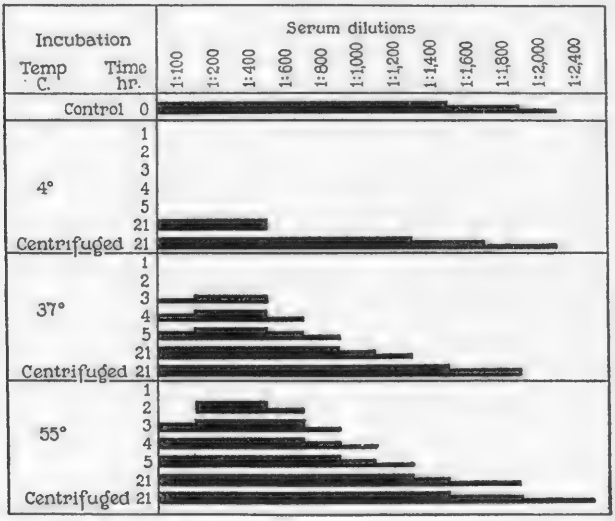
From the readings of spontaneous flocculation obtained in Experiments 3 and 4 before centrifugation of the specimens it was found that the higher temperatures accelerate the flocculation phase of the agglutination reaction also. These readings are indicated in Text-figs. 3 and 4 by cross-hatched areas within the solid black bands.

Although the centrifugation of the incubated specimens of Experiment 3 shows that some absorption had occurred in 16 hours at 21°C. and even in 1 hour at 36°, practically no unaided flocculation resulted at these temperatures in the serum dilutions at which the tests were made. At higher temperatures flocculation appeared earlier and in higher dilutions, but in no instance did it become visible in less than 2 hours, even though the absorption phase had reached its limit in 15 to 30 minutes. Most of the incubation time involved in spontaneous agglutination is required by the relatively slow development of visible flocculation. The influence of temperature upon flocculation is more strikingly shown in the experiment with *Bacillus typhosus*. Here absorption was complete in 15 minutes at 4°C. At this temperature flocculation was not evident for over 2 hours, whereas at 37°C. it appeared in 30 minutes in the lowest dilution examined, and at 55° it appeared in 15 minutes.

In the following experiment with a Type B meningococcus and its homologous antiserum absorption was completed in a short time by exposure to a high temperature and the subsequent flocculation allowed to proceed at various temperatures to determine the relative rate of its development.

Experiment 5.—Type B meningococcus, Strain 1. Homologous monovalent antiserum from a rabbit. Four sets of killed culture suspensions in successive serum dilutions. Heated at 70°C. for 30 minutes. One set centrifuged immediately as a control to determine the completion of the absorption phase. Other sets placed at 4°, 37°, and 55°, and examined at intervals. After 21 hours all the sets were centrifuged to confirm the completion of absorption. The results are given in Text-fig. 5.

These observations on the time and temperature relationships of the flocculation phase reveal the handicap that attends an attempt to study the specific phase of the agglutination reaction with spontaneous flocculation as the indicator. An analysis of these experiments shows that even at high temperatures flocculation does not become visible until long after absorption has progressed to the point required for complete agglutination, while at low temperatures, in high dilutions of the antiserum, flocculation may not appear at



TEXT-FIG. 5. The influence of temperature on meningococcus flocculation.

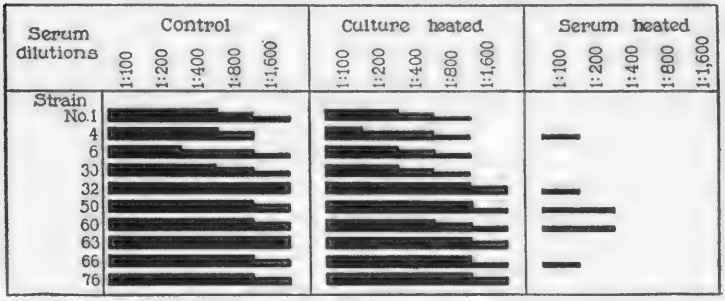
all, even though absorption has advanced appreciably. In order to produce a visible result, spontaneous flocculation requires an absorption reaction several times in excess of that required by the centrifuge method of agglutination.

The Destructive Effect of High Temperatures on Meningococcus Agglutinins.

The decline in agglutination titer which results from prolonged incubation at high temperatures is illustrated in the foregoing

experiments. Such a destructive effect was extensively studied by Eisenberg and Volk (1902), by Joos (1903), by Kraus and Joachim (1904), and by Scheller (1904–05). The following experiment, showing that this deleterious effect is exerted on the serum antibodies rather than on the bacteria, is cited to show the application of the centrifuge method to problems of this character.

Experiment 6.—Year old polyvalent antimeningococcus serum, in successive dilutions, and killed culture suspensions of a number of meningococcus strains, 2 billion per cc., were each divided into three lots and one lot of each was heated at 70°C. for 30 minutes, an incubation period found to have an injurious effect in agglutination tests with these materials. Tests were then set up with mixtures of (*a*) unheated serum and unheated organisms (control); (*b*) unheated serum and



TEXT-FIG. 6. The destructive effect of high temperatures on agglutinins.

heated organisms; and (*c*) heated serum and unheated organisms. These tests were incubated at 55°C. for 4 hours, centrifuged, shaken, and read, with the results shown in Text-fig. 6.

It is seen that the unheated serum and heated cocci react in a manner similar to the controls, whereas the heated serum has lost much of its agglutinating activity. Experiments already cited show that the antigen-antibody complex, after the specific reaction has occurred, is still subject to injury by high temperatures. In Experiment 3, for example, the decline in the agglutination limit on prolonged exposure to 65°, 70°, or 75°C. occurred after the absorption reaction was complete at these temperatures. Tests with antisera which have been stored for some time show that they are particularly

subject to the effects of high temperatures. The typhoid serum used in Experiment 4 was 3 years old, which may account for the decline in the agglutinin titer even at 55°C.

The Relation of Temperature to Mass Action.

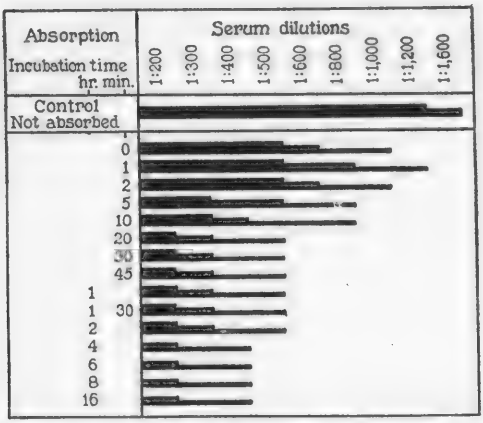
A survey of Text-fig. 3 shows that 0.001 cc. of the antiserum in a volume of 1 cc., a dilution of 1:1,000, contained enough agglutinins completely to clump 1 billion homologous organisms. This complete reaction required more than 4 hours for its development at 55°, but developed in 1 hour at 65° and in 30 minutes at 75°. In lower dilutions, containing an excess of antibodies, a reaction sufficient to clump all the organisms occurred much earlier at these temperatures, and even at 36° and 21° it occurred in the 1:400 dilution in 1 and 4 hours respectively. The presence of an excess of agglutinins greatly accelerates absorption. This fact has long been known, as has the fact that a given bacterial suspension may remove from an excess of serum several times the amount of agglutinins required for its complete flocculation (Eisenberg and Volk[8]). It is interesting to note, however, with the aid of the centrifuge, how quickly the process is brought to its conclusion.

Experiment 7.—Tubes containing 4 billion Type A meningococci, Strain 60, in 2 cc. of homologous antiserum diluted 1:100, were incubated at 55°C. for various periods of time. After centrifugation the clear, supernatant fluids were decanted and diluted successively, mixed with fresh lots of cocci (1 billion organisms in 1 cc. of serum), incubated at 55° for 4 hours, centrifuged, shaken, and read. The results are given in Text-fig. 7.

From the results of the control agglutination it is seen that the serum in a dilution of 1:1,200 originally contained enough antibodies completely to agglutinate 1 billion meningococci, a quantity which may be designated as 1 unit of agglutinin. Before absorption, therefore, each 2 cc. specimen, 1:100, contained 24 agglutinin units. The mere act of centrifugation, without incubation, in contact with 4 billion organisms, deprived the serum of about 12 units of agglutinin, leaving it with a titer of 1:500 to 1:600, so that each billion meningococci removed about 3 agglutinin units. 1 or 2 minutes

[8] Eisenberg, P., and Volk, R., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 155.

incubation did not increase absorption appreciably, but during 5 minutes contact at 55° the serum titer dropped to 1:300, a loss of 18 units, and during 20 minutes at 55° the titer fell to 1:200, indicating the absorption of about 20 units. Each billion meningococci had absorbed about five times the agglutinins necessary for their complete flocculation. This appears to have been about the absorption limit, at this serum dilution, for after 20 minutes only a few more agglutinins were removed from the serum. In other experiments the reaction came to a standstill after 30 and 90 minutes respectively. It is evident that the incubation of absorption tests



TEXT-FIG. 7. Agglutinins left in antimeningococcus serum after absorption at 55°C. with the homologous organisms for various periods of time.

for long periods of time does not result in the removal of correspondingly large quantities of agglutinin. Repeated absorption with fresh bacteria over short incubation periods is the more effective method of removing specific antibodies from serum.

SUMMARY.

The flocculation of bacteria which have absorbed specific agglutinins may be mechanically effected by means of the centrifuge, with results that coincide with those obtained by the standard method of test. Specific serological differences between meningo-

cocci, for example, may be determined by the centrifuge method. The technique is described.

By the elimination of the inconstant time factor in the flocculation phase opportunity is given for a closer analysis of specific absorption, and of the influence of various conditions upon both phases of agglutination.

The velocity of the absorption reaction is a function of the temperature at which it occurs, and the acceleration with increasing temperature is of the order of chemical phenomena. The absorption reaction proceeds most rapidly near the temperature of antibody destruction. The injurious effect of high temperature is revealed first in the serum; the antigen-antibody complex is not less sensitive.

The flocculation phase is also promoted by higher temperature, but lags far behind absorption, and consumes most of the time required for spontaneous agglutination.

The presence of an excess of antibodies greatly accelerates absorption and flocculation. The absorption reaction, under such circumstances, is ordinarily completed within a relatively short time.

THE EFFECT OF PULMONARY CONGESTION ON THE VENTILATION OF THE LUNGS.

By CECIL K. DRINKER, M.D., FRANCIS W. PEABODY, M.D., AND HERRMANN L. BLUMGART, M.D.

(From the Laboratory of Applied Physiology of Harvard Medical School and the Medical Clinic of the Peter Bent Brigham Hospital, Boston.)

PLATES 1 TO 3.

(Received for publication, June 21, 1921.)

Clinical observations have shown that in patients with heart disease the vital capacity of the lungs is frequently less than normal, and that the decrease in the vital capacity bears a close relation to the development of dyspnea. As the tendency to dyspnea increases the vital capacity usually falls, and in patients who are bedridden on account of shortness of breath the vital capacity is rarely more than 30 per cent of the normal. This lowering of the vital capacity expresses the impossibility of increasing the depth of respiration in a normal manner, and under the stress of exercise such subjects are unable to raise the volume of pulmonary ventilation so as to bring about the necessary gas exchange between lungs and blood. The determination of the vital capacity has, therefore, considerable practical significance, since it serves as a guide to the general condition of the patient and often indicates changes in the functional condition of the circulation and respiration that are in harmony with the symptoms but are not necessarily suggested by changes in the physical signs.

The cause of this decrease in the vital capacity of the lungs in heart disease has never been adequately explained. In advanced cases it is, of course, due in part to pulmonary edema, pleural effusion, hepatic enlargement, and similar obvious factors; but in many cases the vital capacity is decreased without any physical signs which account for it, or with physical signs which are certainly insufficient to explain the degree of decrease.

The suggestion has been made (1, 2) that in such cases there may be an increase of pressure in the pulmonary circulation with engorgement of the alveolar capillaries of the lungs. As the alveoli are extremely vascular such a condition might produce a stiffening or *Lungenstarrheit*, in the sense of von Basch (3), which would interfere with their easy expansion and collapse in respiration. There might also be some protrusion of the distended capillaries into the alveoli and thus a decrease in the volume of residual air of the lungs. Since it is impossible to measure pulmonary arterial pressure in human beings, no statements can be made as to the degree of pressure increase which may develop in cardiac disease. From our observations upon cats in which the pulmonary veins have been obstructed, it is apparent that a high degree of pulmonary stasis may be produced without causing much actual increase in pulmonary blood pressure. The lungs act as a slightly elastic sponge, and are able to take up a vast amount of blood without significant pressure change. Furthermore, the normal right ventricle is unable to sustain pressures of any magnitude. We have attempted to increase pressure in the pulmonary artery through a clamp placed upon this vessel just central to its division. It is impossible to adjust such a clamp so as to provide a sustained increase in pressure of more than a few millimeters of mercury in the right ventricle of the cat without causing the death of the animal. We may, therefore, consider that when blood accumulates in the pulmonary circuit with a normal heart there is little attendant pressure increase, and that accompanying changes in ventilation are incident upon volume rather than pressure changes in this circulation.

In chronic cardiac disease with hypertrophy of the right ventricle it is conceivable that pressure in the pulmonary capillaries may reach abnormal levels, but it should be remembered that at the time we are interested in the question—the moment when the vital capacity begins to diminish—the heart muscle is in all probability beginning to fail, and, as a consequence, we are inclined to believe that even in human beings with considerable cardiac hypertrophy the rise in pressure in the pulmonary circuit accompanying a much increased blood volume in this circuit is not great. We are at present engaged upon experiments in which the pressure changes in the pulmonary

artery and capillaries resulting from pulmonary vein compression are being followed, and the statements just made must, therefore, be taken as expressions of our opinion at the moment, not as facts of experimental observation.

Clinical experience contributes several facts which are in harmony with the theory that the interference with the ventilation of the lungs, which shows itself by a decrease in the vital capacity, is due to a chronically increased filling of the pulmonary veins and capillaries. Thus, mitral stenosis is characterized by an early onset of the tendency to dyspnea and an associated low vital capacity. In aortic insufficiency, on the other hand, there is little tendency to dyspnea, and the vital capacity remains high until a relative mitral insufficiency develops and the pulmonary circulation is affected. Again, pleural effusions and pulmonary edema with râles are accepted signs of cardiac weakness. These probably find their cause in congestion of the pulmonary capillaries, and it is logical to suppose that their appearance is preceded by a phase in which the pressure may be very slightly higher than normal, but not yet sufficient to bring about exudation into the alveoli with eventual production of râles or the passage of fluid into the pleural cavity. Before either of these processes takes place there are no physical signs to indicate definitely changes in the pulmonary blood circuit. According to the theory suggested, however, even at this stage the vascular engorgement might interfere with the movements of the lungs and with the size of the alveoli, and cause a decrease in the vital capacity. If this theory is true, its practical significance will be easily understood, for the determination of the vital capacity will give the earliest and most accurate information which can be obtained about the pulmonary circulation. The earliest symptom in most cases of heart disease is an increased tendency to dyspnea and throughout the course of the disease this tendency is usually one of the best guides to the functional efficiency of the heart. It seems quite possible that the development of dyspnea depends largely on the condition of the circulation in the lungs, and, if such be the fact, any method which will help to throw light on one of the most obscure portions of the circulation will be of great clinical importance.

The problem which presents itself, therefore, as the result of the foregoing clinical observations, is to determine whether blood stasis

in the pulmonary circulation produces any effect on the ventilation of the lungs. If such a change does produce a limitation or reduction of the ventilation, then the theory that the reduction of the vital capacity of the lungs in heart disease is due to an increased filling of the pulmonary circulation receives considerable support. It is obvious that the question cannot be decided by the study of patients; the animal experiments to be described in the present communication were, therefore, designed in order to elucidate it.

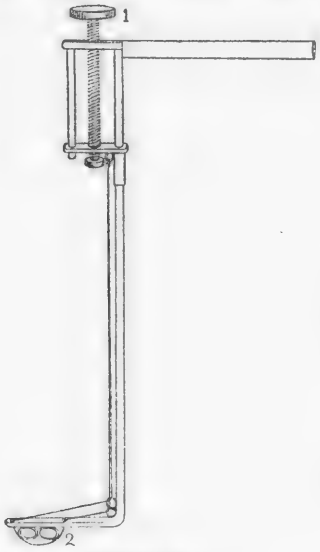
Technique.

The animals selected for study were cats. Because of the exquisite delicacy of the tissues to be studied and the relatively slight displacement in air space to be measured, certain experimental requirements were considered essential.

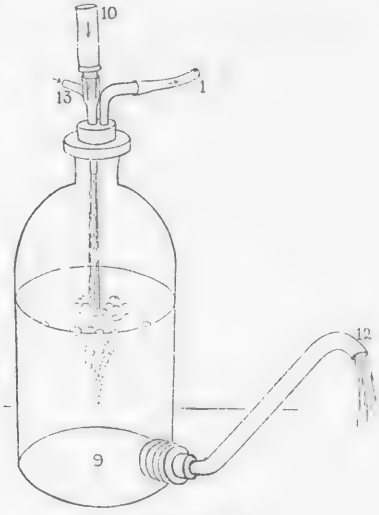
1. All spontaneous and reflex movements of the animal had to be eliminated. Even in the anesthetized animal compression of the pulmonary veins brings about active respiration, and such a reaction interferes with comparative measurements. Urethane anesthesia followed by curare obviates this difficulty.

2. A method had to be devised for compressing and releasing the pulmonary veins without any manipulation of the lungs. This was satisfactorily accomplished by the method described in detail previously (4). An oval section of the chest wall immediately over the heart is removed, the pericardium incised anteriorly, and the cut margins are reflected and sewed to the edges of the chest wall. With proper technique, the pleural cavity is rendered air-tight, permitting for hours independent respiration of the animal. With such a method the heart and great vessels lie exposed *in situ*, resting posteriorly on a pericardial sling. Advantage is then taken of the unusually high reflection of the pericardium in the cat, in which animal a ligature may be slipped about the pulmonary veins without in any way entering the mediastinum. The ligature is then threaded into the clamp as indicated in Text-fig. 1. The pulmonary veins, 2, represented in cross-section, can then be compressed and released by manipulation of thumb-screw 1. We have also found it possible to insert into the pulmonary artery a cannula of the type described by Schafer (5), in order to measure pressure in this artery during pulmonary vein compression.

All of these adjustments can be made after closing the thorax with the pericardium without the use of artificial respiration. In certain instances, of which Experiment 4 is an example, an intrathoracic cannula was inserted and connected either with a water manometer or with a delicately balanced volume recorder. Under such circumstances intrathoracic pressure or lung volume may be followed and the influence of blood in filling up thoracic space noted with accuracy.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Clamp for compression of pulmonary veins.
TEXT-FIG. 2. Apparatus for obtaining constant air pressure.

But in these instances the lungs are invariably collapsed away from the chest wall and are thus able to swell outwardly when they fill with blood. Such a condition is not permitted when the chest wall is properly closed and the lungs are well inflated against it.

3. The air delivered to the animal had to be at a relatively constant pressure and uniform rate of flow. This condition was satisfied by the apparatus illustrated in Text-fig. 2. The water flows down through tubing 10, under a constant head which is maintained by

a bottle suspended 9 feet above, kept full to overflowing. The water passes through an ordinary suction pump, draws in air through by-pass 13, and runs into an air-tight bottle, 9. Here air and water separate. Since the water level in the bottle is determined by the height of the top of the overflow tube, 12, the added amount of water passes out through this overflow tube, while the air is conducted out through tubing 1 to the animal.

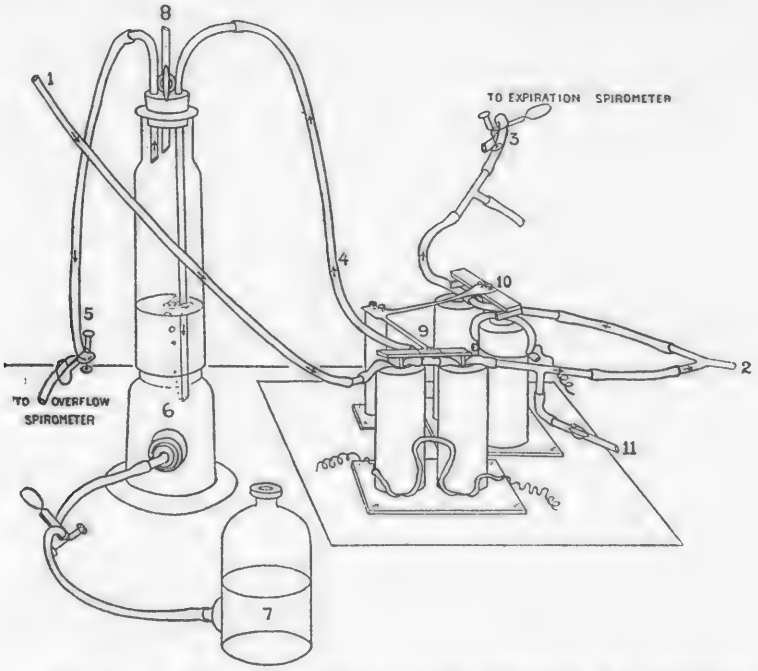
4. The apparatus had to be so designed as to register quantitatively any alteration in pulmonary ventilation. The artificial respiration machine indicated in Text-fig. 3 fulfills this need. It consists of two magnetic interrupters timed by a rotary switch to compress alternately the rubber tubing beneath them. The air passes to the animal from the bottle shown in Text-fig. 2 through an inspiratory tube, 1 (Text-fig. 3), to the cannula, 2, inserted into the trachea of the cat. During the period which constitutes inspiration, one hammer, 9, is raised, while a second hammer, 10, completely occludes the expiratory tube, 3. During expiration, the reverse situation exists, the expiratory air delivered through tube 3 being collected in a sensitive spirometer, known hereafter as the expiration spirometer.

Attention should be drawn to the shunt interposed between the two bars of hammer 9. This shunt, 4, by virtue of its position, operates only during inspiration and is designed to conduct all the air not accommodated by the cat's lungs through bottle 6 to another finely balanced spirometer, termed the overflow spirometer. If the available air space in the lungs is diminished, or the elasticity of the lungs becomes decreased so that they no longer expand as much under the same air pressure, more air will be collected in this overflow spirometer while less air will be collected in the expiration spirometer.

The construction and purpose of bottle 6 interposed in the overflow circuit deserve further comment. The air flows in through tubing 4, bubbles through the water in bottle 6, and escapes to the room through a stop-cock, 8, or, if this be closed and the clamp released, through tubing 5 to the overflow spirometer. Regardless of the avenue of escape of this air, however, the height of the water column in bottle 6, adjustable through container 7, determines the amount of air delivered to the animal. Thus, if the water column is high, little or no air will escape during inspiration into the overflow spirometer and

all the air will pass into the animal to be delivered to the expiration spirometer.

In brief, provision is made to collect all the air delivered to an anesthetized, curarized animal. This air reaches the animal rhythmically but under relatively constant conditions of pressure and flow.



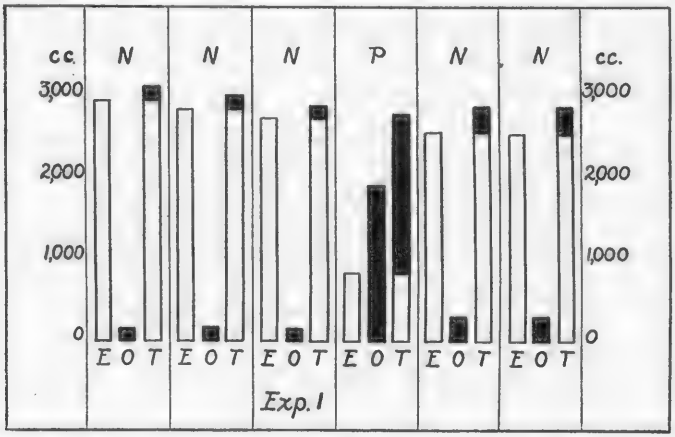
TEXT-FIG. 3. Magnetic interrupter for artificial respiration, with connections to spirometers.

It is collected in two spirometers and the total amount of air which these two receive will check closely in different periods of equal length. But if the pulmonary air space is decreased or if the movements of the lung tissue are restricted, the distribution of the collected air will at once change, a smaller amount being received by the expiration spirometer, and a larger amount by the overflow spirometer.

EXPERIMENTAL.

Experiment 1.—February 7, 1921. Cat; weight 2.8 kilos. 12.05 p.m. 28 cc. of 25 per cent urethane by stomach tube. 3.05 p.m. Operation on thorax completed. 3.30 p.m. 1 cc. of 2 per cent curare intravenously. Cardiometer and pulmonary vein clamp adjusted. Arrangements made for recording rate of respiration and femoral blood pressure.

The further course of this experiment is illustrated by Text-fig. 4 and Fig 1. Text-fig. 4 is a graphic representation of the six collection periods which comprised the experiment. There are, first, three normal periods, labeled *N*, in which air



TEXT-FIG. 4. Graphic representation of Experiment 1. Six 5 minute periods are shown. In this and the following text-figures *N* indicates normal period; *P*, period of pulmonary vein compression; *E*, air collected in expiration spirometer; *O*, air collected in overflow spirometer; *T*, total air collected by both spirometers.

delivered to the animal was collected in the expiration and overflow spirometers without any obstruction of the pulmonary circulation; then one period, labeled *P*, in which the pulmonary veins were compressed by means of the clamp shown in Text-fig. 1; and finally, two more periods, labeled *N*, prior to which the pulmonary vein clamp was released and the animal permitted to return to normal conditions. All periods in this and other experiments were 5 minutes in length, and in Text-figs. 4 to 7 the same method of representation is used. In every case the column marked *E* is the amount of air which actually entered the lungs of the cat and was collected in the expiration spirometer; the column marked *O* is the amount received in the overflow spirometer; and the column marked *T* is the

total amount of air delivered by the respiration apparatus during the 5 minute period, a total obtained by superposing the overflow column upon the expiration column.

Fig. 1 is a kymograph record of conditions during parts of the experiment. Section 2 covers the second period charted in Text-fig. 4. Tracing 1 is the cardiometer record made with the usual type of cardiometer transmitting through air to a large tambour. Tracing 2 is made by means of a second, rather stiff tambour and indicates the rate of respiration. A rubber tube (11, Text-fig. 3) on the inspiratory side of the respiration apparatus leads to this second tambour. Tracing 3 is the blood pressure record taken from the femoral artery by means of a mercury manometer. Tracing 4 is the base-line for blood pressure. Downward marks upon this line indicate 15 second intervals, but in this case are too irregular to be of value since the time-clock was out of order. Upward marks signal various events in the experiment.

During the first three normal periods, a constant amount of air was collected in the overflow spirometer but the total amount was slightly reduced. The change was, however, so much less than that occurring when pressure was applied to the pulmonary veins as to be negligible.

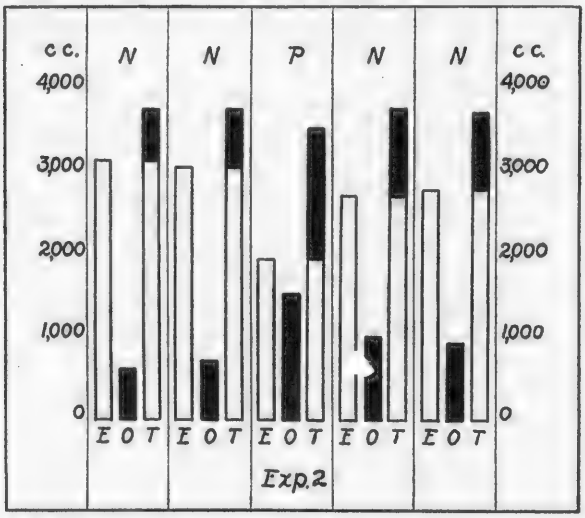
The first upward mark on the base-line in section 4, Fig. 1, indicates the beginning of pulmonary vein compression, brought about by tightening the clamp shown in Text-fig. 1. Between the second and third upward marks in this section of tracing, the air collection shown in *P*, Text-fig. 4, was made, and at the fourth upward mark the pulmonary vein pressure was released. The rise in the cardiometer record during this period shows a certain amount of cardiac dilatation; and the diminished movements of the writing point, a restriction of the individual beats. The fall in blood pressure indicates the degree of interference with filling of the left ventricle. The shift in distribution of the air during this period is clearly shown in *P*, Text-fig. 4. The great reduction in the amount of air collected in the expiration spirometer was due to pulmonary congestion incident upon the pulmonary venous obstruction, and the rise in the overflow collection merely accounts for the air which could not enter the lungs under the conditions imposed.

The two last normal periods *N* and *N*, Text-fig. 4, indicate an almost complete recovery after pulmonary vein release. The fact that this was not absolutely complete probably indicates a certain amount of intraalveolar extravasation which was not at once removed and which occupied space available for air in the first three normal periods. It is, however, clear that a very large measure of recovery did take place, and this means that the changed distribution of air noted in period *P*, Text-fig. 4, was due to intravascular blood.

The lungs, when removed at the close of this experiment, showed narrow longitudinal areas of congestion along the most posterior and consequently dependent portions of each lobe. The degree of change was comparable to that shown in Fig. 5, taken, it is true, from another experiment, but fairly indicative of the pathological changes occurring in experiments accepted by us as picturing the true relation of vascular filling and ventilation. On microscopic examina-

tion one finds epithelial desquamation with red cells and fluid in the alveoli making up the narrow area commented upon in the gross description of the lungs, and normal appearances in the rest of the tissue.

Experiment 2.—February 19, 1921. Cat; weight 3 kilos. 30 cc. of 25 per cent urethane. Operation and preparations for recording similar to those in previous experiment. Text-fig. 5 shows two normal periods, then a period of pulmonary vein compression, and finally, two more normal periods. The result is identical with that noted in Experiment 1. Fig. 2 is a graphic record similar to that in Fig. 1. Section 1 corresponds with the first normal period. Since pulmonary vein compression was produced rather slowly, section 3 has been



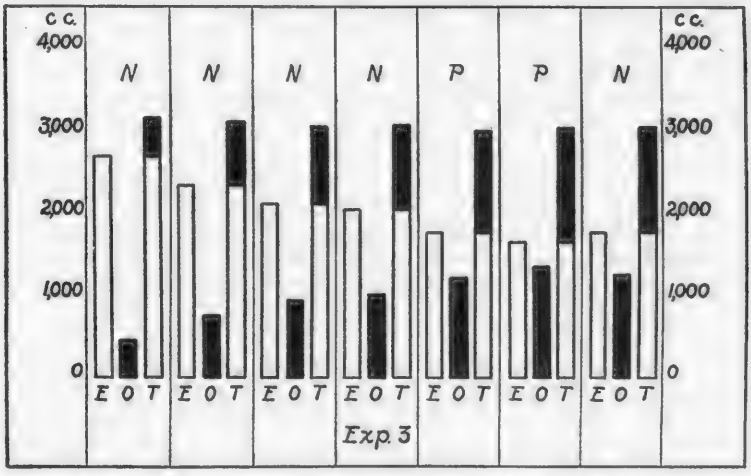
TEXT-FIG. 5. Graphic representation of Experiment 2. Five 5 minute periods are shown.

shortened to save space. The first upward mark on the base-line indicates the beginning of compression. Between the second and third, the air collection noted in *P*, Text-fig. 5, was made, and at the fourth mark the veins were released. Section 4 corresponds with the first normal period *N* after *P* in Text-fig. 5, and displays the condition of the circulation at that time.

On examination of the lungs an area of dark red congestion—not hard nor raised—was noted along the posterior surface of the right lung, and a few similar very small congested areas, chiefly along the margins of the lungs. It is interesting to note that the amount of overflow air in the last collection was somewhat less than in that just preceding, indicating a gradual tendency to return to normal.

In this case, the pulmonary vein clamp was tightened at 12.47 p.m., and collection period *P* took place between 12.56^{30} and 1.01^{30} p.m. The first normal collection period after clamping fell between 1.11^{10} and 1.16^{10} p.m. and the last one between 1.23 and 1.28 p.m.

Experiment 3.—February 19, 1921. Cat; weight 2.9 kilos. 29 cc. of 25 per cent urethane by stomach tube. A technique exactly similar to that of the two preceding experiments was used in this case except that two periods of pulmonary vein compression (*P*, *P*, Text-fig. 6) were employed. The result shows the effect of a progressively weakening heart and the production of extensive pulmonary edema. The heart was unusually large at the beginning of the experiment and



TEXT-FIG. 6. Graphic representation of Experiment 3. Seven 5 minute periods are shown, two of them in this instance being periods of vein compression.

there was more pericardial fluid than usual. Four normal periods were run and the volumes of air collected in the overflow spirometer showed an increase from 473 in the first period to 1,023 cc. in the fourth period. This increase was probably due to the development of pulmonary edema. In the third and fourth normal periods the volume in the overflow spirometer became more constant than it was in the first two, so that light compression of the pulmonary veins was tried in the 5th period.

The condition of the animal at this time is shown in section 5, Fig. 3. Poor adjustment of the cardiometer with leakage explains the atypical cardiometer record. Light compression caused a characteristic effect as noted in the first *P*, Text-fig. 6, and was followed in 9 minutes by a period of greater compression,

the second *P* in Text-fig. 6, and section 6 in Fig. 3. A greater degree of the typical change occurred, and that this was due in part to vascular engorgement, and not wholly to the progressive development of pulmonary edema, is indicated by the fact that release of the vein compression resulted in the last normal period (Text-fig. 6) in a slight return towards normal conditions. Section 7 in Fig. 3 indicates the condition of the animal at this time—a condition of progressively dilating heart and falling arterial pressure, the full extent of which was obscured by a clot which occurred near the end of the tracing.

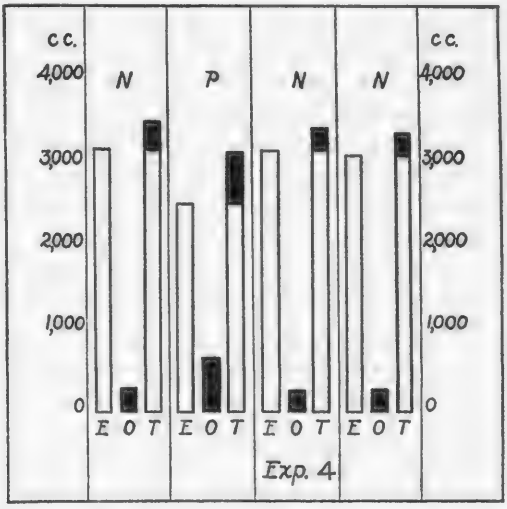
The lungs at the termination of this experiment were in bad condition, over two-thirds of the posterior surface being congested and dark red. There was, thus, extensive permanent damage. The experiment is published in order to display the course of events when pulmonary air space is steadily encroached upon by something more than intravascular blood. Such an animal is perhaps in a similar condition to that of the patient with reduced vital capacity and clear-cut physical signs in the lungs.

Experiment 4.—May 2, 1921. Cat; weight 2.7 kilos. 27 cc. of 25 per cent urethane by stomach tube. All preparations were exactly the same as in preceding experiments with one exception. The line of tracing numbered 2 in Fig. 4 indicates in this instance, intrathoracic volume changes. With the preparation completed in other respects, a glass tube was thrust through the chest wall and tied in place by a purse-string suture. Lung collapse at once occurred, probably bilateral. The intrathoracic cannula was connected with a delicate volume recorder constructed to write on a kymograph. On calibration it was found that a movement of the writing lever of 0.30 cm. recorded a volume change of 1 cm.

Text-fig. 7 is a chart of this experiment and shows a smaller but characteristic result during the period of compression, due probably to the fact that in this case the lungs were free to expand within the thorax, having been collapsed away from the chest wall, whereas in former instances practically all space occupied by blood was alveolar.

Section 1, Fig. 4, shows the condition of the animal during the first normal collection indicated in Text-fig. 7. Section 2 of the same figure is the period of pulmonary vein compression. At the first upward mark on the base-line, tightening of the pulmonary vein clamp was begun. Between the second and third upward marks the air collection period *P* of Text-fig. 7 took place, and at the fourth upward mark the pulmonary vein compression was released. The cardiometer and blood pressure effects are as in former experiments. The tracing of intrathoracic volume rises promptly, indicating a swelling of the lungs with blood, and the individual excursions of the recorder become less owing to less extensive movements of the lungs with each respiration. The maximum rise is equivalent to an encroachment on the intrathoracic space of 6.6 cc. and the individual excursions are diminished by an amount equivalent to 1 cc. Section 4, Fig. 4, represents the conditions during the final normal collection period of Text-fig. 7.

Fig. 5 is a drawing of the posterior aspect of the lungs from this animal. The broken lines lead to darkened sections which indicate the extent of a slight area of congestion—an area not great enough, however, to cause any effect upon ventilation, since it is noticeable that the overflow collections in the last two periods of Text-fig. 7 are not greater than that obtained in the first period of the experiment.



TEXT-FIG. 7. Graphic representation of Experiment 4. Four 5 minute periods are shown.

DISCUSSION.

As indicated at the outset, these experiments were designed to determine whether pulmonary congestion interferes with the entrance of air into the lungs. Congestion was produced by compression of the pulmonary veins at their entrance into the left auricle, and the effect on the air entering the lungs was determined by means of a sensitive artificial respiration apparatus which delivered a constant volume of air with each inspiration. Any obstruction to the entrance of air into the lungs of the animal was indicated by a smaller volume of air being collected on expiration and a larger volume being collected in a spirometer connected with the inspiratory tube, arranged to receive the "overflow" which did not enter the lungs.

The experiments fall into two groups—those in which there was essentially no permanent change in the lungs as a result of the manipulations, and those in which extensive pulmonary congestion with edema into the air passages was produced. In the former, illustrated by Experiments 1, 2, and 4, constriction of the pulmonary veins and the consequent overfilling of the pulmonary circulation caused definite interference with the entrance of air into the lungs. In the different experiments the quantitative effect naturally varied within wide limits. That the effect on pulmonary ventilation was the result of vascular change alone is indicated by the fact that release of pressure on the veins resulted in a practically complete return to the previous normal conditions. The normal appearance of the lungs at the end of the experiments is also evidence that the whole effect was a vascular one, which corrected itself as soon as the obstruction to the circulation was removed.

In the second group of experiments, typified by Experiment 3, compression of the pulmonary veins produced not only the simple vascular effect which appeared in the first experiments, but also caused extensive pulmonary congestion with edema into the air passages. Marked permanent damage was done to the lungs. In these experiments constriction of the pulmonary veins produced an interference with the entrance of air into the lungs, as it did in the first group of experiments, but release of pressure was not followed by a return to normal conditions. The changes in the lungs resulting from the passage of fluid out of the vessels into the pulmonary tissues and the air spaces were not relieved when the pressure in the vessels was allowed to return to normal. In these experiments the blood pressure in the greater circulation fell progressively throughout the observation, and it seems probable that the more extensive and permanent damage to the lungs was in part due to the development of cardiac weakness. This was especially evident in Experiment 3, in which the extensive exudation was undoubtedly due to the combination of circulatory obstruction and weak heart muscle. The results obtained in this second group of experiments are in no way opposed to those obtained in the first group. On the contrary, they supplement them. The two sets of experiments show that if the pulmonary veins are obstructed to such a degree that congestion of the pulmonary

vessels without exudation is produced, there results interference with the entrance of air into the lungs, which is relieved as soon as the obstruction is removed, but that if the circulatory conditions are such that exudation of fluid out of the vessels into tissues and air passages is produced, a permanent interference with the entrance of air into the lungs results.

There appear to be two ways in which pulmonary obstruction can act in order to encroach upon air space in the lung. First, the dilatation of the capillaries may actually take up alveolar space which air could occupy under normal alveolar conditions. This is a simple explanation of the observed change and readily accounts for the fact that less air entered the lungs on each inspiration. When the chest is closed and the lungs expanded so as to fill all the available space. it is clear that extra room which excess blood may occupy can only be obtained at the expense of the alveolar air space. The size of the lungs can only be changed, under these conditions, by pressures great enough to move the chest wall and diaphragm, and it is doubtful whether the right ventricle is capable of accomplishing such a result. When the chest is closed the lungs swell inwardly and large encroachment upon ventilation is the result. This change is illustrated by Experiments 1 and 2. When, however, the chest is open, blood may cause the lungs to swell outward, and under such circumstances the alveolar space may be very little changed and the amount of air entering the lungs not much altered. Experiment 4 illustrates just such a result and brings us to the second method by which pulmonary congestion may reduce the air space. It can be seen that in this experiment the expansibility of the lungs was diminished. The effect is, perhaps, as though the lung were erectile tissue rendered rigid and inelastic through vascular turgescence. Von Basch (3) suggested such an effect many years ago, and Experiment 4 is direct evidence of its existence but under abnormal conditions, the chest in this case being open. We are unable to say in what proportion these two factors operate to reduce ventilation in the closed chest, but are of the opinion that the first is the more important. It is possible that experiments now in progress, in which changes in capillary pressure are being measured, may throw light upon the situation.

We may now consider whether the facts derived from our experiments on animals afford any explanation of the conditions observed in patients with heart disease—conditions which were described at the beginning of this paper. In brief, it has been found clinically that patients with heart disease who become short of breath on exertion are unable to increase the depth of respiration, and that the vital capacity of the lungs is low. Experimentally it has been demonstrated that abnormal amounts of blood in the pulmonary circulation interfere with the entrance of air into the lungs. The clinical condition which has the closest analogy to our experiments is mitral stenosis. They are, indeed, essentially similar, for in mitral stenosis there is an obstruction to the flow of blood in leaving the left auricle, while in our experiments an obstruction was produced to entrance of blood into the left auricle. Both conditions, the clinical and the experimental, produce the same changes in the hemodynamics of the circulation in the lungs. In the early stages of mitral stenosis, when the obstruction to blood flow is slight, there is no abnormal tendency to dyspnea and no decrease in the vital capacity of the lungs. As the degree of stenosis increases, however, a tendency to dyspnea on exertion develops, and the vital capacity of the lungs is less than normal. Physical examination of the lungs reveals nothing to account for this interference with deep respiration. There is no exudation of fluid into the pleural cavities or into the air spaces, and thus no dullness on percussion, no alteration in breath sounds, and no râles. The conditions resemble those in Experiments 1, 2, and 4, in which there was engorgement of the pulmonary vessels with little or no exudation.

The inability to breathe deeply and the low vital capacity, which is one of the outstanding features of mitral stenosis at this stage, may well be explained by engorgement of the pulmonary vessels and lung rigidity, just as these factors explained the interference with the entrance of air into the lungs in our experiments. At a later stage of mitral stenosis, physical examination reveals râles in the air passages and fluid in the pleural cavities. These indicate actual exudation of fluid out of the vessels, and the conditions are similar to those typified by Experiment 3, in which extensive areas of pulmonary congestion were found. In the clinical disease, as well as in the experiments, the development of actual exudation depends on two factors,

the degree of obstruction to blood flow and the weakening of the heart muscle. At this period in clinical cases a still greater fall in vital capacity is found, and a greater tendency to dyspnea. The subject is unable to increase the depth of respiration enough to secure the degree of pulmonary ventilation necessary for aeration of the blood when the needs of the organism for oxygen are increased.

The relation of the conditions in the pulmonary circulation to the production of dyspnea is also suggested by other clinical observations. Mitral stenosis is the disease in which the most immediate effect on the pulmonary circulation would be expected, and dyspnea is an early symptom in mitral stenosis. Mitral insufficiency alone may not produce dyspnea for a long period of time, or apparently not until the heart muscle begins to weaken. The same is true of aortic insufficiency alone, but in association with a mitral lesion and involvement of the pulmonary circulation dyspnea begins to appear.

In aortic stenosis the situation is analogous to mitral stenosis, and when the pressure conditions are transmitted back to the left auricle and the pulmonary circulation, dyspnea becomes a prominent symptom. In all these diseases involving the heart valves the conditions are complicated by a coincident gradual degeneration or weakening of the heart muscle. No completely satisfactory analogy to this exists in our experiment for we were always dealing with a healthy myocardium. It was quite apparent, however, that the weakening of the heart in Experiment 3 acted in the same way as constriction of the pulmonary veins and served to increase the effects due to changes in the pulmonary circulation. It is not at all improbable that weakening of the myocardium alone may be enough to produce the same effects, and there is clinical evidence in harmony with this. Thus, in cases of myocardial weakness in later life the development of dyspnea on exertion is often the first symptom. This is usually accompanied by a fall in vital capacity, but at first there are no physical changes of importance in the lungs. Later, râles and pleural effusion appear. Dyspnea commonly develops early in such cases, while edema of the legs and hepatic enlargement come later. This suggests that in many cases of myocardial weakness the failure of the circulation takes place first in the pulmonary circuit. Clinical experience, as well as the experiments which have been described, indicates that in the

first stages there may be interference with the entrance of air into the lungs without the production of physical signs of lung involvement. At a later stage pulmonary edema and pleural effusion will take place.

SUMMARY.

1. A method is described for producing pulmonary congestion, together with what may be termed a differential spirometer method for studying lung ventilation.

2. The method utilized permits an approximately accurate prediction of degrees of pulmonary edema in the living animal, and suggests avenues of approach for the very difficult problems of pulmonary capillary pressure.

3. It is shown that intravascular blood can encroach markedly upon the pulmonary air space. Although the methods used in these animal experiments do not resemble vital capacity measurements in man, their result is so definite that their applicability to clinical conditions may be considered.

4. The similarity between the experiments described and certain conditions of cardiac decompensation, of which mitral stenosis is the best example, is pointed out.

BIBLIOGRAPHY.

1. Siebeck, R., *Deutsch. Arch. klin. Med.*, 1910, c, 204.
2. Peabody, F. W., *Am. J. Med. Sc.*, 1918, clv, 100.
3. von Basch, S., *Verhandl. deutsch. Cong. inn. Med.*, 1889, viii, 384.
4. Drinker, C. K., *J. Exp. Med.*, 1921, xxxiii, 675.
5. Schafer, E. S., *Quart. J. Exp. Physiol.*, 1918–20, xii, 133.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Kymographic record covering collection periods 2, 4, and 5 of Experiment 1. Upper line of tracing, marked 1, is the cardiometer record; tracing 2, the rate of artificial respiration; tracing 3, arterial pressure, mercury manometer, femoral artery; tracing 4, the base-line for blood pressure. Upward marks on this line signal events referred to in text; downward marks, 15 second intervals—inaccurate in this instance owing to defective clock. The tracing is reduced to about ¼ (it is actually $\frac{64}{275}$) of the original size.

FIG. 2. Kymographic record covering collection periods 1, 3, and 4 of Experiment 2. Lines of tracing similar to those in Fig. 1. The tracing is reduced to about $\frac{1}{4}$ (it is actually $\frac{64}{273}$) of the original size.

PLATE 2.

FIG. 3. Kymographic record covering collection periods 4, 5, 6, and 7 of Experiment 3. Lines of tracing similar to those in Figs. 1 and 2. The tracing is reduced to $\frac{64}{273}$ of the original size.

FIG. 4. Kymographic record covering collection periods 1, 2, and 4 of Experiment 4. Lines of tracing similar to those in Figs. 1 to 3, except that line 2 records intrathoracic volume changes and time marker records 5 second intervals. The tracing is reduced to about $\frac{1}{4}$ (actually it is $\frac{64}{273}$) of the original size.

PLATE 3.

FIG. 5. The lungs of the animal used in Experiment 4. Dotted lines lead to area of congestion described in text.

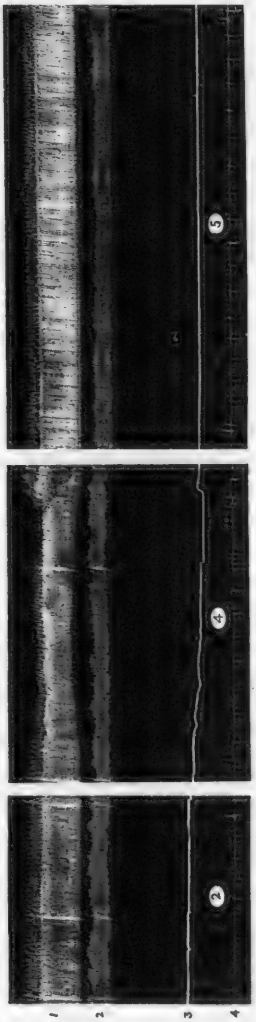


FIG. 1.

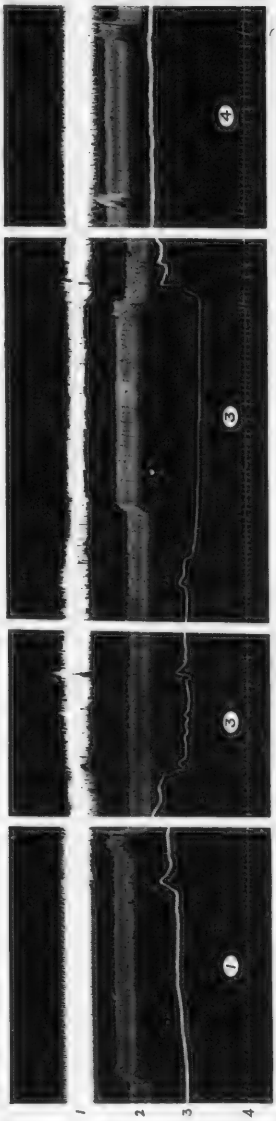


FIG. 2.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)

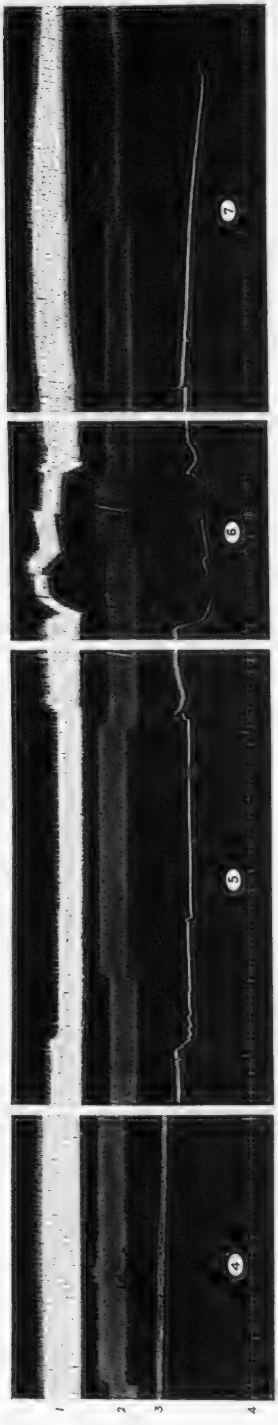


FIG. 3.

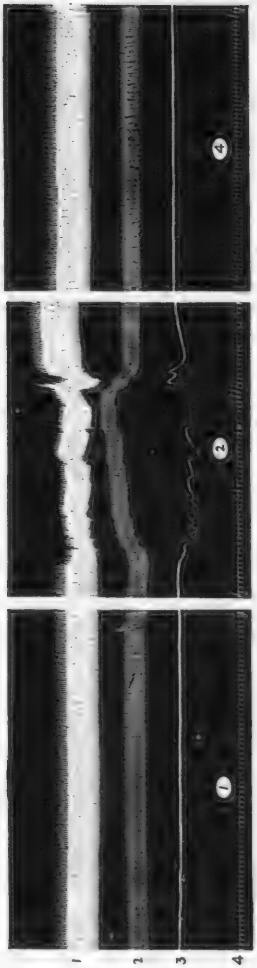


FIG. 4.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)

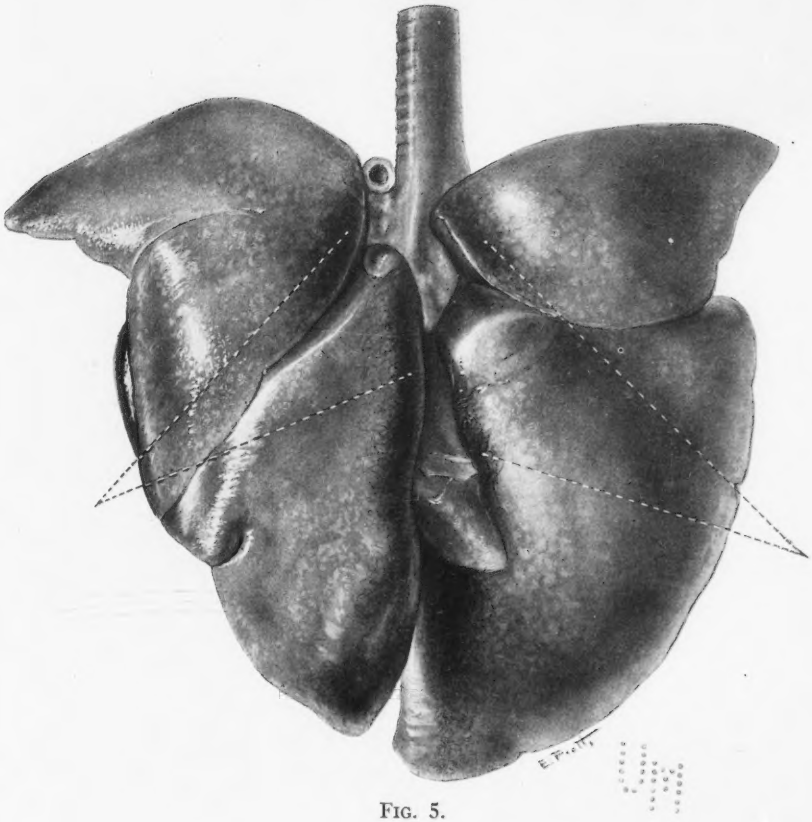


FIG. 5.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)